Machineries for Change: Differentiation in the minimized Eukaryote *Giardia lamblia*

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Machineries for Change: Differentiation in the minimized Eukaryote *Giardia lamblia*

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PhD Thesis

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PART I: Summary

Giardia lamblia (*syn. G. intestinalis, G. duodenalis*) is a protozoan parasite causing diarrheal disease in a wide range of vertebrates and is spread by the fecal-oral route, mainly via contaminated water. Giardiasis causes significant morbidity worldwide and despite treatment infection occasionally leads to a chronic condition. *G. lamblia* has an asexual two-stage life cycle consisting of the cyst in the environment and the proliferating trophozoite in the host`s small intestine. During encystation in the intestine, the flagellated, attachment-competent trophozoite undergoes a significant morphological change into the quiescent cyst encased by an environmentally resistant cyst wall, before being shed into the environment with the feces. This developmental step is essential for the transmission to a new host, which becomes infected after ingestion of only 1 – 5 cysts.

In addition to its public health importance, Giardia has emerged as a promising model organism for cell and also evolutionary biologists. In fact, Giardia could either represent one of the earliest known branches of the eukaryotic lineage, or may have undergone strong reductive evolution and is now more simply organized than the hypothetical last common eukaryotic ancestor (LCEA). Either way, Giardia's highly reduced endomembrane system provides us with an attractive model for protein trafficking studies. Especially, during encystation, a large amount of cyst wall material (CWM) is synthesized and sequestered to a stage-specific secretory pathway for maturation. Importantly, Giardia lacks a typical Golgi apparatus. The presorted CWM is accumulated and delayed in so-called encystation specific vesicles (ESVs) for up to 20 hours until it is finally secreted to the plasma membrane to form a protective extracellular matrix. ESVs share some properties with Golgi cisternae in being post-ER compartments in which cargo is delayed for post translational maturation and processing. One of the major questions addressed in our laboratory is whether ESVs are highly reduced Golgi-remnant organelles, or whether they have evolved separately (convergent evolution) after complete loss of the original Golgi in Giardia. Surprisingly, although most Golgi-associated proteins are missing in Giardia, some conserved factors such as COPI coat proteins associate with ESVs. In this study, we were interested in the function of key factors which recruit coat protein complexes, *i.e.* monomeric GTPases such as giardial Sar1, Rab1 and Arf1. We found that, analogous to higher eukaryotes where Sar1 functions in COPII coated vesicle formation in the early secretory pathway, the Giardia homolog is localized to the ER and, upon expression of a dominant negative Sar1 variant, ESV formation was seriously impaired. The giardial Arf1 homolog was shown to be essential for secretion of the CWM. This is consistent with both Arf1 acting at a late stage of the ~20 hour encystation process and Arf1 function at the *trans*-Golgi in higher eukaryotes. We also found indications that giardial Rab1, similar to its homolog in higher eukaryotes, plays a role in early events of secretory transport and that its complete inactivation impairs ESV formation. Overall, the data developed during my PhD supports the hypothesis that ESVs correspond to a highly reduced Golgi-like organelle subject to developmental regulation.

Giardia cysts are protected by a highly insoluble cyst wall. Surprisingly, only three structural proteins (CWP1-3) and β (1-3)-GalNAc homopolymer have been identified in this biopolymer. The transition from the trophozoite to the cyst stage is induced by as yet undefined environmental cues and leads to a sharp upregulation or induction of CWPs-encoding mRNAs, factors involved in glycan synthesis and a Myb2 transcription factor. *In vitro*, differentiation is triggered by changes in bile concentrations, pH and lipid availability (cholesterol) in the medium. Currently, several distinct but similarly effective protocols are in use. A number of large-scale analyses have been performed to identify the molecular underpinnings of differentiation and its regulation. However, the conditions for induction are not standardized and, more importantly, significant side-effects are predicted for all protocols. Here, I performed a comparative

microarray analysis to determine the full complement of transcriptionally regulated *bona fide* encystation-specific genes. I have shown that the transcriptional response to the most widely-used encystation protocol results in a bipartite response with surprisingly minor involvement of stress genes. Comparing the data from two encystation protocols, as suspected, indeed suggested that a majority of genes in each protocol are induced as a side-effect of culture conditions. I also identified a Myb binding sequence as a signature motif in the short promoter regions of encystation-specific genes. Taken together, the data suggest coordinated regulation of a small set of encystation-specific genes, which mainly encode for structural components of the cyst wall or factors involved in their synthesis and assembly.

In the last part of my thesis I performed the first study in Giardia on the role of stage-specific chromatin remodeling by histone acetylation during encystation. Interestingly, Giardia encodes only one putative histone deacetylase (HDAC) which we could localize to the nucleus. Here, we show that histone acetylation strongly decreases during encystation. Strikingly, a histone deacetylase inhibitor, prevented hypoacetylation after induction of encystation. Nine of the previously identified 18 encystation-specific genes were significantly less expressed and cyst formation was consequently blocked. Consistent with a study suggesting that CWPs are regulated via a repressor bound to its promoter during the trophozoite stage, which is exchanged for an activator bound during encystation, we propose a model in which upregulation of encystation-specific genes during encystation depends on hypoacetylation and consequent silencing of the gene coding for the CWP repressor protein.

PART I: Zusammenfassung

Giardia lamblia (*syn. G. intestinalis, G. duodenalis*) ist ein einzelliger Parasit und weltweit ein Verursacher schwerer Durchfallerkrankungen bei einer Vielzahl von Vertebraten, u.a. dem Menschen. Die Übertragung erfolgt meist durch fäkal kontaminiertes Wasser. Eine Infektion kann trotz Behandlung chronisch verlaufen und bei immunokomprimierten Patienten sogar lebensbedrohend sein. Giardia hat einen direkten Lebenszyklus bestehend aus nur zwei Stadien; der Zyste in der Umwelt und dem Trophozoiten im Dünndarm des Wirtes. Während der Enzystierung im Darm wandelt sich der flagellierte und am Darmepithel adherierende Trophozoit vollständig in die unbewegliche Zyste um, bevor der Parasit über die Fäkalausscheidung in die Umwelt gelangt. Eine höchst umweltrestistente Zystenwand schützt den Parasiten vor Umwelteinflüssen wie zum Beispiel Hitze, Kälte, Trockenheit. Für die Infektion eines neuen Wirtes sind 1 - 5 Zysten ausreichend. Die Enzystierung im Darm ist daher essentiell für das Überleben des Parasiten ausserhalb des Wirtes und für die Wirt zu Wirt Übertragung.

Zusätzlich zu seiner medizinischen Bedeutung ist Giardia ein vielversprechender Modellorganismus für Zell- und Evolutionsbiologen. Giardia scheint nämlich einen insgesamt einfacheren Zellaufbau zu haben, als der Zellaufbau unseres letzten gemeinsamen eukarynontischen Vorfahren (LCEA). Ob dies dadurch beding ist, dass Giardia eine urtümliche eukaryontische Zelllinie vertritt oder ob Giardia eine massive zelluläre Reduktion während der Evolution durchlaufen hat, ist interessanterweise eine Frage die noch nicht geklärt ist. Durch das reduzierte Membransystem ist Giardia jedoch zweifellos ein attraktiver Modelorganismus für die Erforschung der Proteinstransportwege in der eukaryontischen Zelle.

Der Proteintransport ist besonders detailliert beobachtbar während der Enzystierung. Dann wird das Zystenwandmaterial (ZWM) in grossen Mengen im Endoplasmatischen Retikulum (ER) synthetisiert, aussortiert und für die Prozessierung und Reifung in einen stadienspezifischen Proteinsekretionsweg abgesondert. Da Giardia keinen Golgi-Apparat besitzt, wird das Zystenwandmaterial (ZWM) in sogenannten enzystierungsspezifischen Vesikeln (ESVs) akkumuliert. In den ESVs wird das ZWM für mehrere Stunden für die Reifung und Prozessierung gelagert, bevor es schlussendlich an die Plasmamembran sekretiert wird, um die Zystenwand zu bilden.

ESVs besitzen klare Analogien zu den Golgi-Zysternen anderer Eukaryonten, da sie ebenfalls *post*-ER-Kompartimente sind, in welchen Material für die posttranslationelle Reifung und Prozessierung angelagert wird. Die Frage zu klären, ob ESVs Giardia stark reduzierte Golgi-Organellen repräsentieren oder ob ESVs, nach dem vollständigen Verlust eines ursprünglichen Golgi, evolutiv unabhängig entstanden sind (konvergente Evolution) ist ein Hauptziel unsereres Labors. Obschon die meisten typisch golgiassoziierten Proteine in Giardia fehlen, sind interessanterweise einige wichtige Faktoren der Vesikelformation (zum Bsp. COPI, COPII Vesikel) wie zum Bsp. Sar1, Rab1 und Arf1, in Giardia konserviert und mit ESVs assoziiert. Darum haben wir in meinem ersten Projekt die Funktion von diesen drei stark konservierten monomerischen GTPasen (Sar1, Rab1, Arf1) in Giardia untersucht.

GTPasen haben eine Schlüsselfunktion im gesamten Proteinsekretionsapparat der Zelle, speziell in der Formation von Transportvesikeln. Sar1 ist in höheren Eukaryonten an der Bildung von COPII-umhüllten Vesikeln am ER und somit am frühen Sekretionsweg beteiligt. Wir konnten zeigen dass, analog zu höheren Eukaryonten, das homologe Sar1 in Giardia am ER lokalisiert ist und dass durch die Expression dominant negativer Mutanten die ESV-Formation stark beinträchtigt wird. Weiter konnten wir zeigen, dass das homologe Arf1 in Giardia essentiell für die Sekretion des ZWM ist und somit am späten Sekretionsweg des ca. 20 Stunden dauernden Enzystierungsprozess beteiligt ist, analog zu deren Funktion am *trans*-Golgi in höheren Eukaryonten. Auch konnten wir zeigen, dass das in Giardia

homologe Rab1, ähnlich seiner Funktion in höheren Eukaryonten, am frühen Proteintransport und somit an der ESV-Bildung beteiligt ist. Die Analogien zwischen den Funktionen der untersuchten GTPasen in Giardia und in höheren Eukaryoten untermauern klar die Hypothese, dass ESVs stark reduzierte, golgiähnliche Organellen sind, welche stadienspezifisch reguliert sind.

Die Giardia-Zysten sind durch eine höchst wasserunlösliche Zystenwand geschützt. Überraschenderweise konnte man als Komponenten dieser Zystenwand nur drei strukturelle Proteine (CWP1-3) und ein β (1-3)-GalNAc homopolymer nachweisen. Die Faktoren, welche die Enzystierung im Darm induzieren, konnte man bis anhin nicht genau identifizieren. Man konnte jedoch nachweisen, dass die Induktion des Differenzierungsprozesses *in vitro* zu einer stringenten und starken Expression der mRNA der CWPs, der benötigten Faktoren für die Synthese des β (1-3)-GalNAc homopolymers, als auch eines Myb-ähnlichen Transkriptionsfaktors führt. *In vitro* gibt es verschiedene Methoden, um die Differenzierung gezielt zu induzieren. Die Protokolle beruhen im wesentlichen auf einer Erhöhung der Gallen-Konzentration, des pHs und der verfügbaren Lipiden (Cholesterol) im Medium. Obschon mehrere Analysen zur enzystierungsspezifischer Transkription, und der dieser zu Grunde liegenden molekularen Mechanismen ausgeführt wurden, sind die *in vitro* Induktionsbedingungen und mögliche protokollspezifische Nebeneffekte bis jetzt nicht untersucht worden.

In meinem zweiten Projekt haben wir, um die Gesamtheit der enzystierungsspezifisch regulierten Gene zu definieren, eine vergleichende Microarray-Studie durchgeführt, in der wir drei Zeitpunkte der frühen Enzystierung (45min, 3h, 7h) analysierten. Wir konnten zeigen, dass die transkriptionelle Reaktion, induziert durch das meist gebrauchte *in vitro* Protokoll, eine zeitlich zweigeteilte Regulation der Gene auslöst und dass diese transkriptionelle Reaktion auf die Enzystierung überraschendwerweise kaum einer stressinduzierten transkriptionellen Reaktion gleicht. Des Weiteren haben wir zwei *in vitro* Induktionsmethoden verglichen und konnten zeigen, dass wie vermutet ein Grossteil der Gene kultivierungsbedingt induziert wird. Die Expression dieser Gene scheint daher eher ein protokollspezifischer Nebeneffekt zu sein, als dass diese Gene *bona fide* enzystierungsspezifische Gene sind. Mit einer bioinformatischen Analyse konnten wir in allen Promoter-Regionen der enzystierungsspezifischen Gene eine Myb bindende Sequenz identifizieren und als Charakteristik der Promoter dieser Gene definieren. Unsere Daten deuten somit klar darauf hin, dass ein minimales Set an enzystierungsspezifischen Genen, welche hauptsächlich für die strukturellen Komponenten der Zystenwand und Faktoren für deren Synthese kodieren, koordiniert reguliert ist.

Mein drittes Projekt umfasst eine Analyse der stadienspezifischen Genexpressionsregulation durch Histone-acetylierung und somit Chromatinstrukturveränderungen während der Differenzierung in Giardia. Giardia kodiert interessanterweise nur eine Histone-Deacetylase (HDAC) im Genom und wir konnten zeigen, dass diese im Zellkern lokalisiert ist. Im Weiteren konnten wir eine klare Abnahme der Histone-acetylierung während der Enzystierung nachweisen. Durch die Zugabe eines HDAC-Inhibitor zu den Zellen konnten wir diese Abnahme verhindern. Auffallenderweise waren dann neun der 18 enzystierungsspezifischen Gene signifikant weniger reguliert und die Zystenbildung wurde durch den Inhibitor ganz geblockt. Eine externe Studie legt nahe, dass ein zu den CWP-promotorbindender Repressor während der Enzystierung durch einen CWP-promoterbindenden Aktivator ausgetauscht wird und so die CWP-Expression reguliert wird. Folgerichtig schlagen wir das Modell vor, dass die Regulation der *bona fide* enzystierungsspezifischen Gene darauf beruht, dass die für den Repressor kodierende Region während der Enzystierung hypoacetyliert wird und somit die Expression des Repressors unterbunden wird.

PART II: Aim of the thesis

Giardia has a simple two stage life cycle consisting of a motile trophozoite stage, which proliferates in the host`s small intestine, and a environmentally resistant cyst. Trophozoites transform into dormant cysts in ~24 h in vitro. This developmental step is essential for the transmission to a new host which gets infected after ingestion of cysts from the environment.

The significant morphological change from the flagellated, attachment-competent trophopzoite to the oval cyst covered by a thick cyst wall is controlled by the stringent transcriptional regulation of encystation-specific genes. In addition to a general downregulation of cellular functions, specific environmental triggers induce the synthesis of large amounts of cyst wall material (CWM). The CWM is sorted to a stage-specific regulated secretory pathway after synthesis in the ER. Secretory protein trafficking in Giardia occurs without a steady state Golgi apparatus which normally is at the center of the eukaryotic secretory system.

A combination of strong cell polarity, drastically minimized cellular systems and a small genome, makes Giardia a unique model system to study fundamental biological processes like transcriptional regulation, protein trafficking and cellular differentiation.

The aims of my thesis were to investigate the basis and the scope of changes in gene expression in encysting Giardia parasites. In addition, I explored the molecular underpinnings of regulated secretory transport of cyst wall material during stage- differentiation.

PART III: Introduction

1. *Giardia lamblia*

1.1. Giardiasis

Giardia lamblia (*syn. G. intestinalis, G. duodenalis*) is a protozoan parasite that infects a wide range of vertebrates. The parasite is spread by the fecal-oral route, mainly via contaminated water. The prevalence in humans ranges from 2 - 7 % in developed countries to 20 – 30 % in countries with low quality drinking water [1,2]. In the livestock industry, giardiasis is associated with significant economical losses due to decreased productivity [3]. Despite its impact, giardiasis is a poorly understood disease. Human and animal giardiasis causes significant morbidity worldwide [4,5]. Frequently observed and typical symptoms of giardiasis, such as diarrhea, malabsorption and weight loss [1,5] are caused by proliferating Giarida trophozoites that inhabit the proximal portion of the small intestine. There is no general consensus on the pathogenesis [1], especially because Giardia trophozoites are not invasive and no toxins have been identified in the secreted fraction. Despite the availability of several effective drugs for treatment, such as 5-nitroimidazole compounds [6], treatment failures have long been recognized and cases of chronic diarrhea and malnutrition, especially also in immunocompromised individuals and children, occur frequently and can even be life threatening [1,4]. Children in particular might also suffer serious consequences, including retarded growth and development, and poor cognitive function[7,8].

1.2. Giardia life cycle

G. lamblia has an asexual two-stage life cycle. The motile, flagellated trophozoite inhabits the lumen and epithelia of the small bowel of the vertebrate host. Cysts, in contrast, are a quiescent, environmentally resistant form that survives for several months in cold water. After ingestion by a new host, the cyst re-activates and escapes its shell upon arriving in the intestine. This process is triggered by stomach acids and aided by host proteases, which, together with proteases secreted by the parasite, leads to a break-down of the cyst wall [9]. After exposure to biliary fluids, some trophozoites form cysts in the intestine before being shed into the environment by the host. The exact trigger of encystation *in vivo* is unknown, however. *In vitro*, encystation can be induced by raising the pH to 7.8 and adding bile salts and lactic acid [10], or by depleting cholesterol in the growth medium [11].

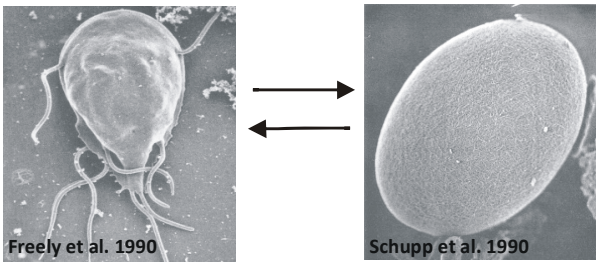

Figure 1: *Giardia lamblia*
The parasite cycles between a flagellated trophozoite (left) inhabiting the host and the oval shaped cyst (right) in the environment.

1.3 The Giardia cyst

Despite the high environmental resistance of cysts, the cyst wall appears to be of surprisingly low complexity: three paralogous cyst wall protein family members (CWP1-3) and a β(1-3)-GalNAc homopolymer have been identified as the major cyst wall components [12-17]. While the manner of integration of the glycan with CWPs into the extracellular matrix is unknown, a membrane-anchored type 1 cysteine-rich protein (HCNCp), which localizes to the plasma membrane of cyst forms [18], could function as a possible link between the cyst wall and the cell surface. The precursor of the GalNAc polymer is UDP-GalNAc, whose enzymatic synthesis pathway from glucose scavenged from the medium is upregulated during encystation [19-23]. The final polymer has two “sharp” ends that can both be

covalently linked to proteins [22], and it reacts poorly with known lectins [24]. Characteristics of CWPs are a common domain structure with a central leucin-rich repeat necessary for incorporation into the cyst wall, and an N-terminal domain that is indispensable for correct targeting to ESVs [14,25]. Only CWP2 possesses a C-terminal extension rich in basic amino acids. This extension is partially cleaved off by a cysteine protease during secretory transport and before incorporation into the cyst wall (*Gl*CP2) [14,26,27]. This and other modification processes contribute to the formation of a highly resistant biopolymer which protects the parasite in the environment and during stomach passage.

1.4 Giardia cell biology

The endomembrane system of the vegetative, half-pear shaped trophozoite form (7 - 12 µm, see Figure 1) of *Giardia* is much simpler compared with that of a typical eukaryotic cell. In particular, Giardia lacks organelles, such as a classical Golgi, mitochondria, peroxisomes and lysosomes. Further, Giardia lacks almost the entire machinery for addition of N-linked glycans to secreted proteins in the endoplasmatic reticulum (ER). Enzymes that add mannose and glucose to a dolichol precursor are absent. Thus Giardia Asn-linked glycans are unusually short and consist of two added GlcNAc residues at the most, which are not further modified during secretory transport. Consistent with this, Giardia has no machinery for N-glycan dependent quality control of protein folding and degradation [28,29], which is present in higher eukaryotes [30-32].

The Giardia trophozoite contains two nuclei harboring nucleoli [33], it has a glycogen-rich cytoplasm, acidified peripheral vacuoles (PVs), and a labyrinthine tubular-vesicular network (TVN), segments of which are decorated with ribosomes, consistent with rough ER [34] (see Figure 2). Functionally, the entire TVN is most likely ER, which extends bilaterally through the cell and is continuous with the nuclear envelopes. PVs are small (150-200 nm) organelles that localize directly under the dorsal plasma membrane, and in a small area at the center of the central ventral disk [1,35] which serves as an attachment organelle. PVs are thought to be endosome-lysosome-like compartments. More likely, these organelles which are acidified and contain proteases and nucleases serve to digest bulk endocytosed material before trafficking to the TVN [34]. A recent study showed that the Giardia ER may be a pluripotent compartment that serves as a site of protein synthesis, as well as for degradation of endocytosed material imported from the extracellular milieu [34].

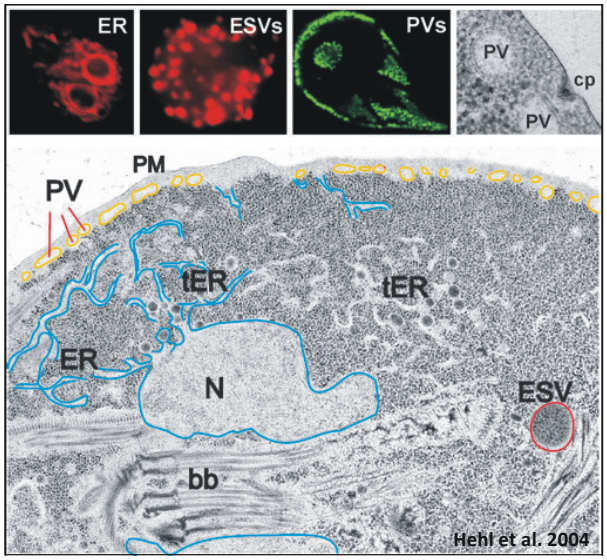

Figure 2: Compartment morphology in trophozoites and encysting Giardia
The upper panels show the ER with nuclear envelope (red), ESVs (red) and PVs (green). Right: morphology of PVs, the plasma membrane and a coated pit structure (cp).
Bottom: transmission EM image of an early encysting cell showing ER, transitional ER (ER exit) sites with small (a 60 nm) transport vesicles and tubular membranes, one small ESV and peripheral vesicles (PVs, arrows). A part of the reticular ER composed of mostly rough ER including the nuclear envelope, PVs and the ESV have been traced blue, yellow and red, respectively, to enhance their appearance. N, nucleus; PM, plasma membrane; bb, basal bodies.

1.5 Giardia as a model organism

In addition to its public health importance, Giardia has become an extremely interesting model organism for cell and evolutionary biologists. The field is currently split with some researchers maintaining that Giardia represents one of the earliest known branches of the eukaryotic lineage [1,36,37], while others argue that Giardia has undergone strong reductive evolution and is now simpler in many ways than a hypothetical last common eukaryotic ancestor (LCEA) thought to have existed ~1-1.5 billion years ago [38,39]. The exact position of the Diplomonads on the phylogenetic tree remains to be determined, however [40,41]. Therefore, the absence of organelles or specific functions can be interpreted either a primary basic feature, or as a consequence of secondary reductive evolution. In the case of the Giardia mitochondria it is clear that Giardia can be viewed as a secondary amitochondriate [1,42]. Small, double membrane bounded mitosomes clearly represent relic mitochondrial organelles [42-45]. The presence of mitochondria or derived organelles in all extant eukaryotes suggests that the origin of mitochondria pre-dates the LCEA [38]. Protists with long independent evolution and highly modified organelles offer ideal models for the study of mitochondrial evolution [45]. Giardia also possesses a very basic trafficking machinery that, interestingly, lacks a classical Golgi organelle and Golgi functions which are predicted for the LCEA [38]. Importantly, even though the trafficking machinery of Giardia appears to be much simpler than the one of higher eukaryotes, and lacks familiar morphological landmarks and functions, some elements are highly conserved (mentioned in detail in 2.1 and 2.2 below). Until recently, it was generally accepted that Giardia replicates only asexually by binary fission. However, recent molecular analyses challenge the idea that Giardia is a strictly clonal organism, and there is genetic evidence that homologous recombination occurred between two Giardia assemblages [46]. In addition, genetic data indicate that some genes with possible function in meiotic recombination [47] are present in Giardia, although evidence for this has not been obtained [48-50]. To date, there is no direct experimental evidence for a sexual life cycle in Giardia. Meiosis appears to have evolved early in the history of eukaryotes (approx. 850 million years ago [51]). However, exactly how and when this happened remains a basic and unanswered question in cell biology.

Giardia is also a versatile laboratory model due to its amenability to reverse genetics methods [52] and its simple two-stage life cycle that can be completed *in vitro* [10,11,53]. Giardia can serve as a model for other parasites with similar life cycles *e.g.* Entamoeba [54], which produce environmentally resistant and infectious stages as well. Furthermore, its small ~12 Mbp (~6500 predicted genes) genome is fully sequenced [49]. Deep sequencing technology allows rapid comparison between different *G. lamblia* assemblages, as previously shown [55], and whole genome microarrays are made freely available by the J. C. Venter Institute, allowing system-wide analysis of the transcriptome of the cell. In addition, large-scale approaches including identification of the glycoprotein complement [24] and mitosome-associated factors [42] by mass spectrometry have been performed.

2. Encystation

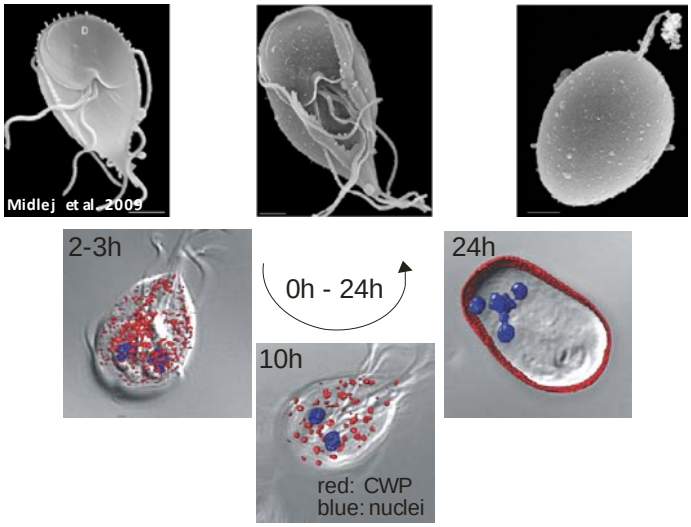

Figure 3: Giardia encystation

Upper panel: Scanning electron microscopy pictures of Giardia reveals a gradual change from the flattened dorso-ventral pear shaped trophozoite to the round oval cyst during encystations (scale bars 1μm).

Lower panel: cyst wall protein (red) is synthesized in the ER, accumulated into encystation specific vesicles (ESVs) and secreted to the plasma to form the cyst wall in approximately 24 h.

2.1 Constitutive and regulated protein secretion in *Giardia lamblia*

One of the main lines of research in our laboratory is the investigation of the molecular mechanisms of secretory protein sorting, transport and targeting during the life cycle of Giardia. Thus far, two export pathways for secretory proteins have been described. One constitutively active pathway supplies the trophozoite plasma membrane with transmembrane-anchored variant-specific surface proteins (VSPs) (see Figure 3). VSPs are highly immunogenic surface antigens which form a uniform surface coat covering the entire trophozoite, including the ventral disk and the flagella. The VSP coat provides a protective interface between the parasite and the environment in the host gut [56,57]. Only one of the ~200 VSPs encoded in the genome is expressed at any time by trophozoites, and antigenic variation, *i.e.* switching to another VSP copy is thought to be essential for host immune evasion and appears to be regulated by RNA interference [58,59]. Surface exposed VSPs are continuously turned over by proteolytic cleavage, shedding and secretion of newly synthesized protein [60].

The main focus of my thesis work, however, is on regulated secretory transport of cyst wall material (CWM) in Giardia (see Figure 4). During encystation the cell generates a completely new secretory organelle system which is dedicated to the production and maturation of the large amounts of cyst wall material exported to the cyst wall. Specifically, newly synthesized CWPs are sorted away from constitutively secreted cargo as they are exported from the ER [61]. The presorted CWM is then accumulated and delayed in encystation specific vesicles (ESVs) (see Figure 3 and 4) for many hours until it is finally secreted to the plasma membrane to form a protective extracellular matrix (see Figure 3). In contrast to higher eukaryotes where delay of secretory cargo for

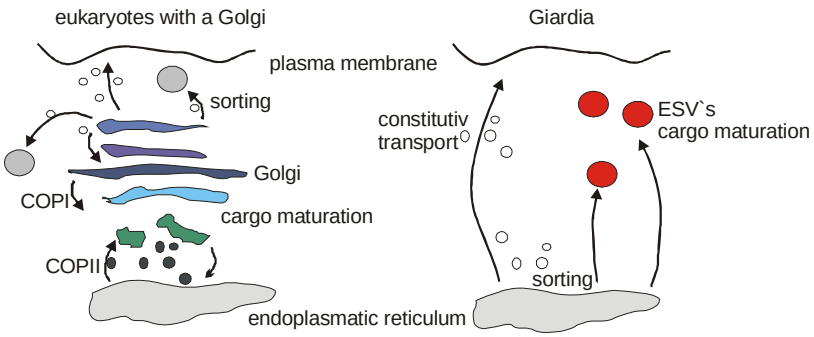

Figure 4: Schematic of the protein secretory pathway of a eukaryotic cell with a stacked Golgi (left) and the Giardia cell (right).

post translational maturation and sorting to different destinations occurs in the Golgi, Giardia secretes constitutively exported proteins with little or no modification directly from the ER.

Secretory transport in higher eukaryotes is organized by an intricate and highly dynamic membrane system with a Golgi apparatus at the center. This organelle which was first described more than a century ago can assume different morphologies, e.g. in most cells a classical stack of cisternae with distinct *cis* to *trans* polarity at the cell center, or delocalized, but biochemically distinct cisternae, or ministacks closely associated with ER exit sites. In all cases, however, anterograde transport of cargo begins with export of newly synthesized secretory proteins in transport intermediates from the ER to the *cis* most compartment of the Golgi. This is a key step involving a quality control step to assure correct folding of secreted proteins which are then sequestered for export to COPII coated vesicles, ER export sites [62,63]. The core components of the COPII coat protein complex are the small Ras-like GTPase Sar1, recruited by sec12 in the ER membrane, the sec23/sec24 subunits which interact with cytoplasmically exposed cargo determinants and form the prebudding complex on the ER membrane. The sec13p/sec31p subcomplexes [64] finally achieve deformation of the vesicle membrane which leads to it pinching off and release from the ER. COPII vesicle budding and export from the ER normally, but not always, occurs at specialized ER domains, also called transitional ER (tER) [65,66]. COPII transport intermediates uncoat after GTP hydrolysis in Sar1 and fuse homotypically to form an ER-to-Golgi intermediate compartments (ERGIC) in mammalian cells. This matures to become the *cis*-Golgi membrane cisterna [67,68]. Vesicle fusion for ERGIC formation is dependent on the Rab1 GTPase [69], which is recruited to transport vesicles and regulates vesicle targeting and fusion with receptor compartments. Rab GTPases act synergistically with tethering factors and soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) [70,71]. Once proteins reach the Golgi, they pass through the cisternae and undergo a series of post translational maturation processes before final sorting and secretion to the plasma membrane and other endomembrane organelles, such as lysosomes and peroxisomes [67]. Transport carriers at the *trans*-Golgi network, the main sorting station of the secretory pathway, are formed by clathrin coats and adaptor protein complexes (AP1, AP3). The small GTPase Arf1 functions as a key regulator in these steps [72] in addition to regulating formation of COPI-coated vesicles [73,74]. COPI-coated carriers are thought to be responsible for intra Golgi and retrograde transport. There are two models to explain the dynamics of Golgi cisternae and cargo transport from the *cis* to *trans* Golgi [75]. Briefly, while the cisternal maturation model posits that each Golgi cisterna matures as it migrates outward through the stack carrying the cargo with it [76-78], (i.e. the cargo never leaves the cisterna), the vesicular transport models posits that the Golgi has a stable cisternal structure through which cargo is transported by small COPI coated vesicular carriers that bud off the rims and fuse with the next cisternae [76].

In Giardia no compartment which could be identified as a Golgi has been detected. The only instance where secretory cargo is delayed after export from the ER is in encysting cells, which accumulate pre sorted cyst wall material in large compartments, called encystation-specific vesicles (ESVs). In contrast to a Golgi in higher eukaryotes, ESVs are not steady state organelles but arise *de novo* during encystation and are individual large saccular compartments that have no morphological similarity to the stacked Golgi of higher eukaryotes. Although no modifying enzymes which normally define Golgi cisternae biochemically are present in ESVs, these organelles share some properties with Golgi compartments [79-81]. ESVs are post-ER organelles in which cargo is delayed for post translational maturation and processing ([82-84] Konrad *et al*. PLoS Pathogens, in press). Interestingly, they are sensitive to brefeldin A, a fungal metabolite that inhibits Arf1 function and induces disassembly of the Golgi. Although the central organelle of secretory transport is missing in Giardia, its secretory system,

consisting mostly of an elaborate ER, is still typically eukaryotic. In a previous study, 39 members of protein families involved in budding and fusion of coated transport intermediates were identified: Two adaptor protein complexes (APs), COPI (coatomer), COPII, an exceptionally small number of GTPases (six Rabs, two Arfs, Sar1), and seven SNARE proteins [80]. In addition, colocalization of transitional ER regions and early ESVs with coat protein COPII, and recruitment of COPI and clathrin to maturing ESVs strongly suggested that ESVs form by fusion of ER-derived vesicles containing CWM, analogous to the classical ERGIC/*cis*-Golgi [83]. A subsequent study using an organelle-specific proteomics approach to discover additional ESV-associated proteins showed that the chaperone HSP70/Bip cycles through ESVs [79].

As described above, small monomeric Ras-like GTPases, such as Sar1, Arf1 and Rab1, act as molecular switches in the secretory pathway of the eukaryotic cell organizing budding and fusion events. Arf,Sar and four Rab GTPases are well conserved in Giardia, whereas the other Rab proteins are highly divergent [79,83]. Nevertheless, because small GTPases act in a compartment-specific manner they are useful markers for the characterization of compartment organization in the secretory pathway. In general, GTPases cycle between an active, GTP-bound and an inactive GDP-bound stage and direct vesicle budding and fusion. Consistent with the function of Sar1 in COPII-coated vesicle formation at the ER exit site in higher eukaryotes, the giardial Sar1 orthologue was localized to the ER/nuclear envelope structure [61]. In addition, a giardial homolog of the Ypt1/Rab1-GTPases [85] has been localized to early ESV membranes [79]. Rab proteins constitute the largest family of small GTPases and are involved centrally in ensuring that cargoes are delivered to their correct destinations, therefore, they control membrane identity and vesicle budding, uncoating, motility and fusion through the recruitment of effector proteins [86]. Rab proteins are associated with many effectors, i.e. exchange of GDP with GTP is catalyzed by guanine nucleotide exchange factors (GEFs), which recognize specific residues in the switch regions and facilitate GDP release. While conversion from GTP- to GDP-bound stage form occurs through GTP hydrolysis catalyzed by GTPase activating proteins (GAPs). As many as 38 different human Rab GAPs with restricted specificity have been identified. Interestingly, Rab proteins appear to be highly conserved in function among extant eukaryotes [87], and a special feature is that there is often a direct structure-function linkage among species. Interestingly, Giardia harbors a minimal number of only six Rabs [80], while approximately 29 Rabs are present in *Caenorhabtitis elegans* and 11 in *Sacharomyces cerevisiae* and over 60 in humans [69,87].

Taken together, the data on secretory transport in Giardia which were generated during almost 20 years of research by many different groups are consistent with ESVs corresponding to highly reduced Golgi-like organelles which have become stage-specifically regulated. Despite the significant and sometimes fundamental differences between ESVs and a classical Golgi, our working model for ESV neogenesis and maturation during stage differentiation has many intriguing analogies to the cisternal maturation model of the classical Golgi ([79,84,88] Konrad *et al*. PLoS Pathogens, in press).

2.3 Gene transcription during encystation in *Giardia lamblia*

While many of the conserved factors involved in the trafficking machinery of Giardia, such as Rab1, Sar1, and Arf1, are constitutively expressed [80], encystation-specific genes, *e.g.*, *CWP*1-3, encoding structural components of the cyst wall, are transcriptionally silent in the trophozoite stage. Following the induction of encystation, transcripts of all three CWPs can be detected within 2-3 h post-induction (p.i.). The maximum expression level was detected at around 7 h p. i. [25,89]. Thus far, only single encystation-specific genes, such as genes encoding structural proteins (cwp1-3) and key enzymes of the cyst wall

polysaccharide biosynthesis pathway have been described [14,15,18,23,25,90], but a systematic effort to identify the full complement has not been successful.

Interestingly, expression control of all encystation-specific genes described so far occurs at the transcriptional level, and very short upstream sequences are sufficient to confer stage-specificity to reporters in transgenic cells. This is consistent with the finding that Giardia promoter regions are very short (about 50-100 bp). There is no TATA box, in contrast to promoters in higher eukaryotes [91], but an A/T-rich region, directly surrounding the transcription start side of the gene, can act as an initiator (Inr) element [49,92]. The 80 bp region upstream of the cwp1-3 genes share a *cis*-acting regulatory element (C(T/A)ACAG) described as a Myb-binding site [93]. A Myb2-like protein is one of the few transcription factors identified in Giardia and is also known to be upregulated during encystation [93-95]. DNA-binding analysis showed that Myb2 appears to modulate its own transcription as well as that of cwp1-3 and a glucosamine 6-phosphate deaminase [93,95]. However, the literature does not quite agree on the sequence of the Myb binding motif since another study showed that in a random site selection experiment, the oligonucleotides bound by Myb2 contained a conserved sequence of GTTT(G/T)(G/T) [95]. Recently, a plant-like WRKY was shown to be an important transactivator of the *cwp1-2* genes during Giardia encystation [96]. Similarly, an ARID/Bright like transcription factor [97] was postulated to be involved in stage regulation [98]. In addition, the promoter region of *CWP*2 was analyzed in detail using deletion studies within 64 bp upstream the start codon of the gene [89]. Deletion of elements in -23 to -64 significantly increased expression of a reporter construct in vegetative trophozoites, suggesting that this area contains a gene silencing *cis*-acting element. Deletion in -23 to -10 still (containing no overlap with the conserved Inr region) decreased expression in encysting cells, suggesting that this region may contain gene transcription activating *cis*-acting elements. These results indicate that Giardia uses (a) repressor mechanism(s) for tight, encystation-specific control of the *CWP*2 promoter [89]. Taken together, the data suggest that stage-specific control of encystation-specific genes is achieved by multiple mechanisms which include modulators of basic transcriptional activity, enhancement and de-repression, with different contributions of several *trans*-acting factors or *cis*-acting elements.

2.3 Regulation of gene expression in *Giardia lamblia*

Transcriptional upregulation of encystation specific genes such as CWP1-3 and Myb2 [25,93] is essential for successful stage conversion. Remarkably, only a few (~9) transcriptional factors have been identified in Giardia compared to Entamoeba (>50) and Trichomonas (>160) [99], suggesting that additional gene expression management systems underlie development changes. For example, detection of microRNA (miRNA) precursors (snoRNAs) has provided clear evidence that miRNAs [100] play a role in RNA silencing in Giardia and specifically, that they have a potential role in regulating VSP expression and antigenic variation [101]. As mentioned above, another study suggested that VSPs regulation occurs also over small RNAs but by a different mechanism (RNA interference) in Giardia [59,102]. However, taken together, those findings are clear evidence that transcript regulation via small RNAs occurs in Giardia but which mechanism exactly is present, still remains to be determined.

A special characteristic of Giardia transcripts are the short 5` - untranslated region (5`- UTR). For the translational initiation, in higher eukaryotes, the 5`-UTR of an mRNA plays an important role and a minimal length of about 20 nucleotides is required to prevent leaky ribosome scanning. In Giardia, however, the mRNAs have 5`UTRs mostly in the range of 0 to 14 nucleotides without a conserved sequence and despite this short 5`UTR it was shown that 5`capping occurs [49,103-105]. Interestingly, the 5`-UTR of Giardia mRNAs can be reduced to a single nucleotide before the translational start codon

without affecting its translation efficiency or causing leakiness. Overall, mRNAs were shown to possess a uniquely simple capping structure at the 5` UTR, which provides an optimal translation efficiency, suggesting a protein synthetic machinery that may bridge the gap between prokaryotes and the advanced eukaryotes [103].

One of the major determinants of gene expression patterns in eukaryotes is chromatin structure which regulates access of transcription factors to DNA. Chromatin is primarily composed of nucleosomes, consisting of the DNA doublestrand tightly wrapped around a core of four histone proteins (H2A/H2B and H3/H4). The *N*-terminal domains of the histones extend outward and can be post-translationally modified (*e.g.,* methylated, acetylated and phosphorylated). In particular, histone acetylation appears to be ubiquitous among eukaryotes [106-108] and plays an important role in regulating gene expression by modifying DNA packaging. Histone hyperacetylation results in chromatin decondensation and high accessibility to the transcription machinery. Conversely, hypoacetylation leads to a tighter DNA-histone binding, with consequent chromatin condensation and gene silencing. A breakthrough in the investigation of histone modifiying enzymes in protozoa was the discovery and functional analysis of a highly conserved histone acetyl transferase in the free-living ciliated protist *Tetrahymena thermophila.* The study implied that these histone modifying proteins are linked to regulatory transcriptional mechanisms and have an ancient origin and are therefore likely to be operational also in other protozoa [106]. In Apicomplexa (*e.g*., Toxoplasma, Plasmodium) and also in Entamoeba [109], histone modifications, particularly acetylation, are emerging as key regulators of parasite differentiation and stage conversion [107]. For both, Toxoplasma and Entamoeba, the specific role of their histone deacetylases (HDACs) was investigated and it was proposed that HDAC-dependent regulation of specific genetic loci is stage-specific.

Again consistent with a general minimization of systems in Giardia a highly reduced histone acetylation machinery was found to be present in Giardia and first described in the study included herein, harboring only one but a highly conserved histone deacetylase. In contrast Toxoplasma, has six putative HDACs in which belong to three classes [110]. Further, recent findings have proposed that acetylation of histone lysine residues correlates with the expression pattern of a specific VSP gene [111], suggesting a role for acetylation-deacetylation processes in Giardia gene expression regulation.

3. Goals of the thesis

3.1 Project 1: Conserved factors of the trafficking machinery for protein sorting and export

Controlled secretion of the cyst wall material to build a protective extracellular matrix is essential for transmission of Giardia to a new host. Despite the absence of a conventional Golgi in Giardia, proteins are sorted during encystation and accumulated in ESVs. One of the major question addressed in this and previous studies was whether ESVs are highly reduced Golgi remnant organelles, or whether they have evolved separately after complete loss of the original Golgi in Giardia. In this study, we focused on the cellular localization and function of conserved key factors (*i.e.* monomeric GTPases) of the secretory pathway of *G. lamblia*.

We addressed the following questions: i) What are the functions of the conserved small GTPases in export and secretion of the cyst wall material? ii) To what extent do ESVs show analogies to *cis*-Golgi compartments? And iii) are ESV interconnected and do ESVs exchange cargo? We hypothesized that cargo exchange could be a means to synchronize protein maturation among the ~30 ESVs in a cell.

3.2 Project 2: Transcriptional profiling of encystation

Several previous studies have investigated the transcriptional induction of single genes during encystation (see 2.3). The trigger(s) of induction of encystation *in vivo* is unknown, but several different *in vitro* encystation protocols were established. System-wide transcriptional analysis of encysting parasites out of the gut of a host could most probably give the most accurate view on transcriptionally regulated genes during encystation. However, those *in vivo* studies are not feasible because the parasites are not easily accessible and would require synchronized induction of encystation. As an alternative approach we performed two separate transcriptional profiles of encysting parasites, each induced with a different but equally effective *in vitro* protocol. The rationale was that the conditions for induction of differentiation generated a lot of unspecific effects. Thus, by building an intersecting dataset with two different protocols we would be able to filter out the unspecific signals and obtain the set of *bona fide* encystation genes. Using this approach we tested the hypothesis that induction of encystation *in vitro* activates the transcription of a very limited set of genes coding for proteins directly required for stage differentiation. Importantly, we provide the first study comparing two different *in vitro* encystation protocols to differentiate between side effects of a particular encystation protocol applied and genes which are really involved in the encystation process.

In this study, we addressed the following questions: i) Which and how many genes are upregulated during encystation? ii) Is the transcriptional response time-dependent? iii) Can we detect additional structural components of the cyst wall? And iv) can we predict possible common regulatory elements among encystation-specific genes?

3.3 Project 3: The role of histone acetylation in stage conversion

Accessibility of *trans*-acting elements to DNA is an important regulator of transcriptional activation/silencing of genes, and is often determined by the chromatin structure. There is increasing evidence that chromatin remodeling through histone acetylation plays a key-role in stage specific gene expression in protozoan parasites [106,107,109]. Here, we investigated the role of histone acetylation-deacetylation in the Giardia life cycle for the first time. In this study, we showed that the overall rate of histone acetylation decreases during encystation. Based on this result, we tested the hypothesis that a change in histone acetylation and concomitant chromatin condensation plays a major role in Giardia stage differentiation and regulation of encystation-specific genes.

Here, we addressed the following questions: i) To what extend is the histone acetylation machinery conserved in *G. lamblia*? ii) Can we localize key enzymes of the histone acetylation machinery in *G. lamblia*? iii) What happens when we inhibit the histone deacetylation process during encystation? And iv) is histone acetylation/deacetylation a key mechanism of transcriptional regulation of encystation-specific genes?

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PART IV

Manuscripts

1) Neogenesis and maturation of transient Golgi-like cisternae in a simple eukaryote

Sasa Stefanic*, **Laura Morf***, Caroline Kulangara, Attila Regös, Sabrina Sonda, Elisabeth Schraner, Cornelia Spycher, Peter Wild and Adrian B. Hehl
**These authors contributed equally to this work,* accepted 23 April 2009, Journal of Cell Science 122

My contribution to this work consisted of the localization study and functional analysis of the giardial Rab1 GTPase in ESV- and cyst formation during stage conversion (see page 27 – 29, Figure 3 and 4). I joined this project at the start of my PhD and I was subsequently involved in all steps of the project and the manuscript development.

Neogenesis and maturation of transient Golgi-like cisternae in a simple eukaryote

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Summary

The highly reduced protozoan parasite *Giardia lamblia* has minimal machinery for cellular processes such as protein trafficking. *Giardia* trophozoites maintain diverse and regulated secretory pathways but lack an identifiable Golgi complex. During differentiation to cysts, however, they produce specialized compartments termed encystation-specific vesicles (ESVs). ESVs are hypothesized to be unique developmentally regulated Golgi-like organelles dedicated to maturation and export of pre-sorted cyst wall proteins. Here we present a functional analysis of this unusual compartment by direct interference with the functions of the small GTPases Sar1, Rab1 and Arf1. Conditional expression of dominant-negative variants revealed an essential role of Sar1 in early events of organelle neogenesis, whilst inhibition of Arf1 uncoupled morphological changes and cell cycle progression from extracellular matrix export. The latter led to development of 'naked cysts', which lacked water resistance and thus infectivity. Time-lapse microscopy and photobleaching experiments showed that putative Golgi-like cisternae in *Giardia* develop into a network capable of exchanging soluble cargo at a high rate via dynamic, tubular connections, presumably to synchronize maturation. The minimized and naturally pulsed trafficking machinery for export of the cyst wall biopolymer in *Giardia* is a simple model for investigating basic principles of neogenesis and maturation of Golgi compartments.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/122/16/2846/DC1

Key words: *Giardia*, Golgi, Regulated secretion, Membrane transport, GTPase, Arf1, Sar1, Cyst wall, Membrane tubule

Introduction

Giardia lamblia (Adam, 2001), an intestinal parasite with worldwide distribution, is a unique model organism to investigate relic organelles (Tovar et al., 2003) and minimal cellular mechanisms (Morrison et al., 2007). Minimal systems in *Giardia* are believed to be mostly the result of reductive processes associated with a parasitic lifestyle (Lloyd and Harris, 2002), but might also reflect some evolutionary basic characteristics (Morrison et al., 2007). The membrane transport system responsible for constitutive and regulated export of surface molecules along multiple pathways lacks an identifiable Golgi complex (Marti et al., 2003a). Consistent with this, most of the machinery for biosynthesis of core N-glycans and all associated quality control modules for endoplasmic reticulum (ER) export are absent (Robbins and Samuelson, 2005; Samuelson et al., 2005). Despite the completely different organization of the trafficking system, a minimal set of universally conserved molecules has been identified (Dacks et al., 2003; Elias et al., 2008; Marti et al., 2003b), suggesting that the basic principles of membrane transport are conserved. During stage-differentiation to infectious cysts, trophozoites synthesize large amounts of cyst wall material (CWM), which is secreted to the surface of the cells in a regulated fashion to form a protective extracellular matrix. The giardial CWM appears to be of very low complexity: only three paralogous cyst wall proteins (CWP1-CWP3) and a simple β1-3 GalNAc homopolymer (Gillin et al., 1996; Jarroll et al., 2001; Lujan et al., 1995b; Sun et al., 2003) polymerize to a highly effective biological barrier on the surface of encysting cells. An epitope-tagged, membrane-anchored high cysteine non-variant cyst protein (HCNCp) (Davids et al., 2006) had been localized to the periphery of encysted cells, possibly at the interface between the plasma membrane and the cyst wall. In vitro, encystation is triggered by environmental cues, for example, low cholesterol concentration and elevated pH (Lujan et al., 1996), leading to de-repression of the genes coding for CWPs (Davis-Hayman et al., 2003). In the ER, CWPs are rapidly sorted from other secretory cargo and partitioned into newly emerging organelles, dubbed encystation-specific vesicles (ESVs), which have no equivalent in proliferating trophozoites (Lujan et al., 1995a; Marti and Hehl, 2003). Encystation takes 20-24 hours in vitro, and ESVs develop to approximately uniform size in the first 8-10 hours, after which they mature and become secretion competent (Marti et al., 2003a). In the absence of constitutive and inherited compartments that could be defined as Golgi cisternae in *Giardia*, ESVs are the only post-ER delay compartments to accommodate the machinery and the cargo for the post-translational maturation processes (Touz et al., 2002) required to produce the CWM. This is supported by circumstantial evidence, such as sensitivity to brefeldin A and recruitment of COPI components and other markers of the secretory system on ESV membranes (Lujan et al., 1995a; Marti et al., 2003b), but direct evidence that ESVs are Golgi analogs is lacking.

The classical Golgi complex is a dynamic organelle system at the center of the secretory pathway in all eukaryotic cells from basal single-celled eukaryotes to mammals (Warren and Malhotra, 1998). In unicellular organisms, Golgi organization is less complex, ranging from unlinked stacks associated with ER exit sites in *Pichia pastoris* (Bevis et al., 2002; Rossanese et al., 1999) and several protozoa (Benchimol et al., 2001; Hager et al., 1999; Struck et al., 2008), to individual, functionally distinct Golgi cisternae without stacked organization in *Saccharomyces cerevisiae* (Rossanese et al., 1999). Microsporidia, intracellular fungal parasites of mammals, have highly reduced trafficking systems, and lack a conventional Golgi (Beznoussenko et al., 2007).

Our working model for ESV neogenesis and maturation during encystation stresses the analogy to the cisternal progression model (Losev et al., 2006), with the important difference that ESVs are not steady-state organelles but arise in response to a pulse of CWM exported from the ER (Marti and Hehl, 2003; Marti et al., 2003a). The process following ESV genesis can be understood as a simultaneous progression (maturation) of ESVs from a *cis* to a *trans* stage concluding with regulated exocytosis of the CWM. Another tenet of this model is that ESV neogenesis has similar requirements as Golgi reconstitution upon mitotic exit in higher eukaryotes or Golgi neogenesis in some protozoa (He et al., 2004). Because both are dependent on membrane transport from the ER to the Golgi, a prediction is that key elements of the early secretory machinery, such as the small Ras-family GTPases Sar1 and Arf1, coat proteins and factors, such as SNAREs, that direct membrane fusion events, are required for ESV formation. Association of a few of these components with compartments of the regulated secretory pathway in *Giardia* has been demonstrated previously (Marti et al., 2003b).

To test the hypothesis that ESVs are minimal, stage-regulated Golgi analogs we investigated the machinery involved in regulated export and maturation of the CWM using a functional approach. The alternative possibility is that ESV genesis is independent of classical ER export machineries but directed by a non-conserved mechanism specific to *Giardia* and linked to the biophysical properties of the CWM, for example a propensity to self-aggregate in ER subdomains. As a first step, we tested whether the genetically conserved key elements of the early secretory pathway – the giardial homologs of the small-GTPases Arf1, Sar1 and Rab1 – are necessary for establishing the regulated pathway for CWM export. We show that the functional requirements for ESV formation are surprisingly conserved despite fundamental differences in compartment organization supporting the analogy of the process to neogenesis of *cis*-Golgi cisternae. We then addressed the question of how maturation of the apparently highly condensed CWM is coordinated among ~30 ESVs. Surprisingly, quantitative analysis of ESV cargo demonstrated that the CWM in mature ESVs is highly fluid and polymerizes only on the cell surface. In addition, analyses of organelle dynamics in living cells revealed that ESVs are not isolated individual organelles, but establish transient physical connections, which allow rapid exchange of CWM.

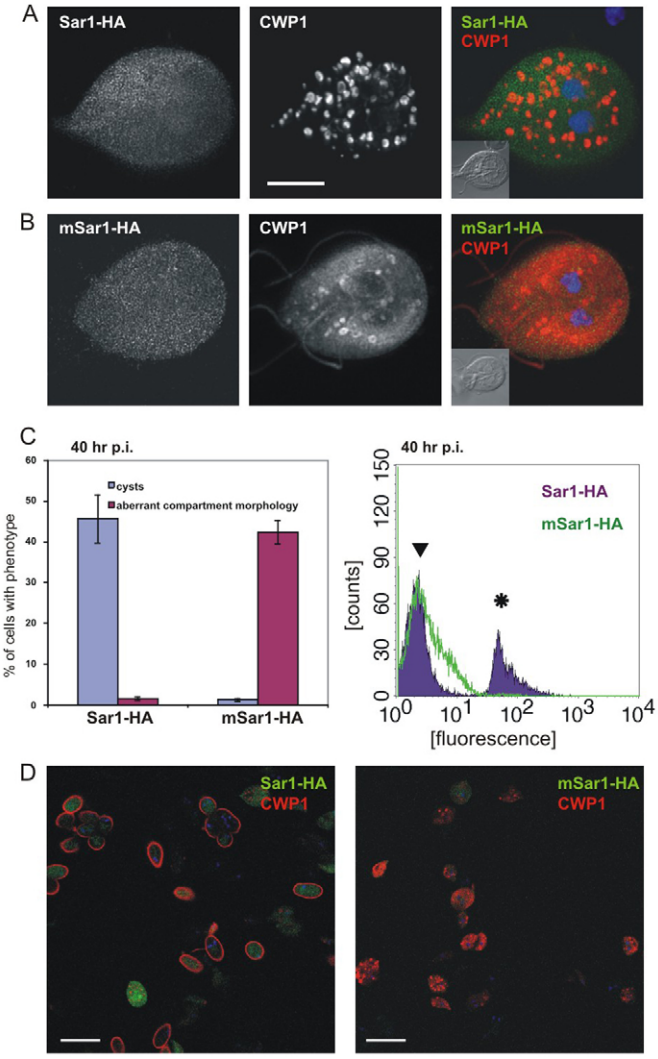

Fig. 1. Giardial Sar1 is necessary for normal ESV development and cyst formation. (A,B) Confocal microscopy of representative cells at 8 h.p.i. expressing high levels of wild-type or mutant (H74G) HA-tagged giardial Sar1 (green). ESVs were detected with a monoclonal antibody against CWP1 (red). Cells expressing the dominant-negative mSar1-HA show aberrant ESV morphology and unspecific secretion of CWP1 to the surface of the trophozoites. Insets: differential interference contrast (DIC) images. Nuclear DNA was stained with DAPI (blue). Scale bar: 5 µm. (C) Left panel: quantification of cyst yields and ESV morphology in cell lines expressing Sar1-HA (control) or mSar1-HA at 40 h.p.i. (endpoint). Results are shown as a percentage (mean ± s.e.m.) of total encysting cells. n=10; P<0.009. FACS analysis of surface-exposed CWP1 in cell populations labeled with anti-CWP1 confirmed manual counts is shown on the right. Note the absence of the cyst peak (asterisk) in mSar1-HA line. Encysting trophozoites (arrowhead). (D) Comparative fluorescence microscopy analysis of detergent-permeabilized cells expressing Sar1-HA or mSar1-HA at 24 h.p.i. Cyst development was abolished in virtually all cells expressing mSar1-HA. Scale bars: 20 µm.

Results

Sar1 function is necessary for ESV and cyst formation

The conserved giardial Sar1 GTPase localizes to ER membranes and putative ER-exit sites in encysting *Giardia* (Marti et al., 2003a), suggesting the involvement of the COPII coat complex in ER export of CWM and establishment of ESV. We tested whether Sar1 was necessary for ER-to-ESV transport in *Giardia* by conditional expression of a dominant-negative variant to generate a Sar1 knockdown. The rationale for choosing this more direct approach rather than mRNA ablation (see also below) was the <20 hour window of opportunity afforded by the only inducible system yielding strong ectopic gene expression currently available (Davis-Hayman and Nash, 2002; Hehl et al., 2000). A short *CWP1* promoter element controls transcription of a gene of interest and is induced in differentiating trophozoites (for direct comparisons of induced

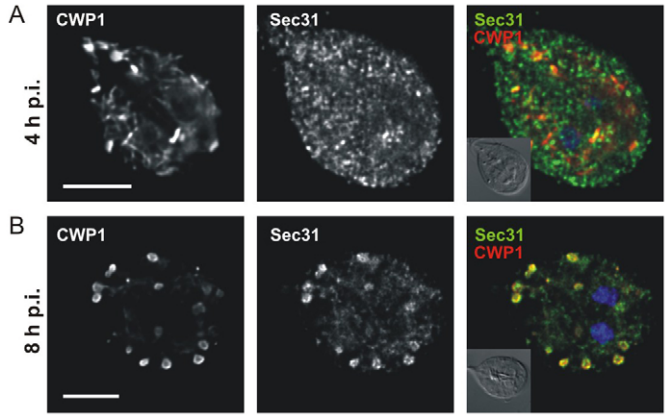

Fig. 2. Localization of the giardial COPII subunit Sec31. Confocal immunofluorescence microscopy analysis of wild-type cells. Specific polyclonal antibodies showed recruitment of COPII coat complex to regions with emerging ESVs at early stages of encystation, 2-4 h.p.i. (A) and 8 h.p.i. (B). Total projections of image stacks are shown. Nuclear DNA is stained with DAPI (blue in merged images). Insets show DIC image. Scale bars: 5 μm.

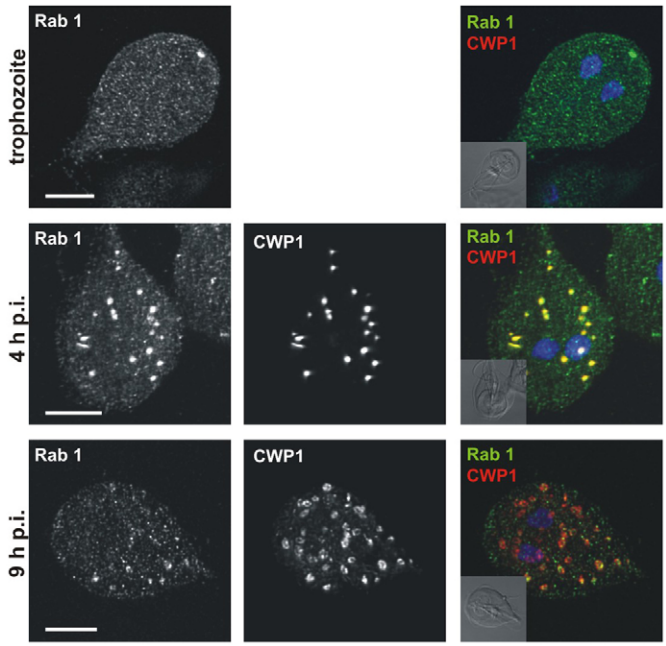

Fig. 3. Giardial Rab1 associates transiently with developing ESVs. Developmentally regulated subcellular localization of Rab1 in proliferating and encysting trophozoites analyzed by confocal microscopy using specific antibodies against the recombinant protein. Rab1 is recruited from a cytoplasmic localization in trophozoites (non induced, top row) to ESV membranes in the first 8 h.p.i. of encystation. In the second phase of encystation (>8 h.p.i.) this association disappears and most of the Rab1 signal is again detected in the cytoplasm. Images show maximum projections of deconvolved image stacks. Insets show DIC image. Scale bars: 5 μm.

recombinant and endogenous GTPases, see supplementary material Fig. S4C). Thus, differentiation, CWM synthesis and export were induced simultaneously with the potentially interfering dominant-negative factor. This is only successful if the effect takes hold early enough to interfere with trafficking. Expression of a *Haemophilus influenza* hemagglutinin (HA)-tagged, mutant Sar1 (mSar1-HA, H74G) with this system resulted in almost complete block of ESV and cyst formation (Fig. 1). Expression of CWPs was induced, but the protein appeared to be dispersed in the ER, in compartments with atypical morphology, and was frequently mistargeted to the surface of trophozoites (Fig. 1B). Quantitative analysis and microscopy confirmed that only ~2% converted to cysts, whereas cells expressing the corresponding wild-type Sar1 showed normal encystation efficiency (~45%) (Fig. 1C,D). This underscored the importance of Sar1-dependent ER export for ESV and cyst formation, and was strong indirect evidence for a crucial role of the conserved COPII heterotetramer in ER export of CWM. Indeed, confocal laser-scanning microscopy (CLSM) analysis of chemically fixed cells using a specific antibody against a giardial Sec31 homolog (supplementary material Fig. S6) showed that this COPII subunit was recruited from the punctate cytoplasmic localization to the vicinity of emerging ESVs (Fig. 2). Similarly to Rab1 (see below), Sec31 lost this specific association as encystation progressed beyond 8 hours post-induction (h.p.i.).

Giardial Rab1 has a role in ESV and cyst wall formation

A role for Sar1/COPII in the export of CWM to ESVs suggested that other conserved key factors of the proximal secretory pathway might be involved in neogenesis of ESVs. A giardial homolog of Yip1 (Heidtman et al., 2003) has previously been localized to early ESV membranes (Stefanic et al., 2006). In higher eukaryotes, this multipass membrane protein was shown to be involved in membrane recruitment of di-prenylated Rab proteins (Yang et al., 1998). Using antibodies against the highly conserved giardial Rab1 (supplementary material Fig. S6), we detected this GTPase transiently on ESV membranes, with maximal recruitment before 8 h.p.i. of encystation (Fig. 3). Similarly to Sec31, Rab1 lost this association in the second half of the 20-24 hour encystation process (Fig. 3), consistent with a function in early secretory transport of CWM and establishment of ESV organelles.

To test the role of Rab1 in encystation, we used conditional expression of an N-terminally tagged 'GTP-locked' mRab1-HA (Q68L). In contrast to cells expressing mSar1-HA, the observed encystation phenotype was not as clear-cut. We observed a significant number of encysting trophozoites (8 h.p.i.) with dispersed CWP signal in immunofluorescence microscopy (Fig. 4A, top row), and a minor proportion (~1-3%) of morphologically normal cysts (i.e. with four nuclei, an oval shape and resorbed flagella) with a strongly reduced or completely undetectable CWP1 signal on the surface (Fig. 4A). The lack of internal CWP1 signal in these cysts suggested that CWM had been secreted or degraded, but was not deposited on the surface. Quantitative analysis of surface-exposed CWP1 on the population level (Fig. 4B) revealed a minor but distinct shift of the cyst peak to the left in cells expressing mRab1-HA compared with the population expressing Rab1-HA, indicating depletion of CWP1 on the surface.

As an independent approach to interfere with Rab1 function during encystation, we attempted ablation of Rab1 mRNA. RNAi in Giardia is controversial (Saraiya and Wang, 2008) and gives inconsistent results. Conditional expression of a Rab1 antisense RNA under the control of the strong CWP1 promoter had no effect (data not shown). However, expression of a Rab1-specific RNA stem-loop (Fig. 4C; supplementary material Fig. S2B,C) resulted in moderately reduced (60-70%) Rab1 mRNA level by quantitative RT-PCR at 4 h.p.i., compared with a slight upregulation in control cells (Fig. 4C). Interestingly, an identical cyst phenotype was

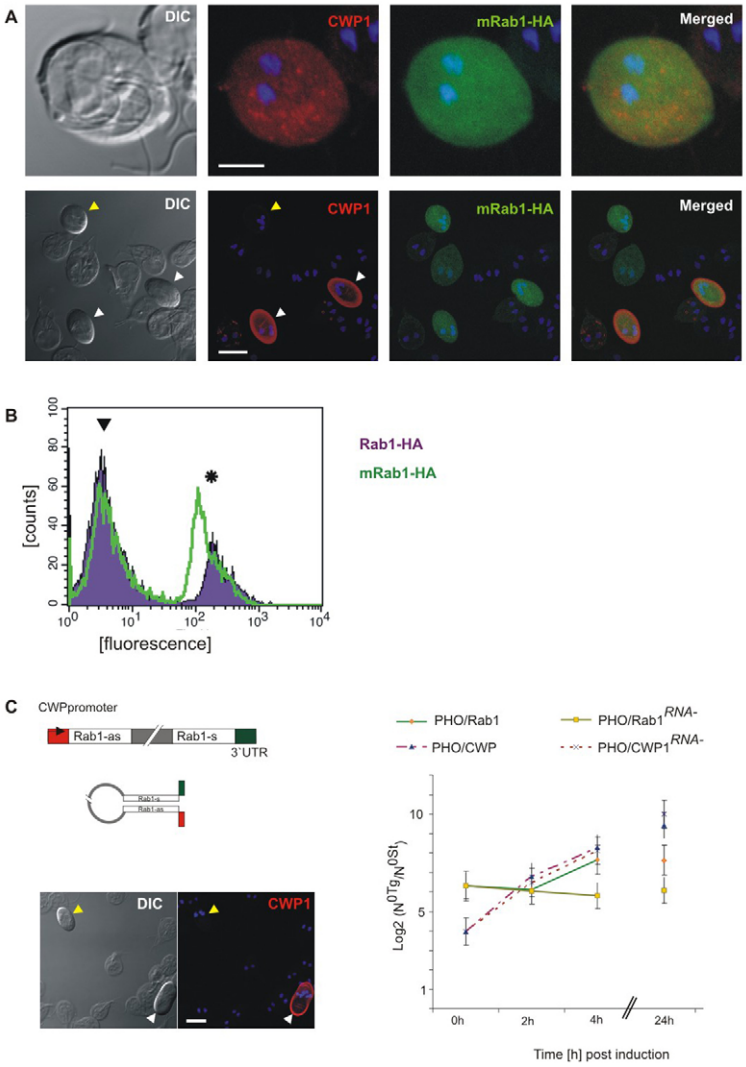

Fig. 4. Rab1 is necessary for ESV development and cyst wall formation. (A) Immunofluorescence microscopy analysis of chemically fixed and detergent-permeabilized cells overexpressing the dominant-negative (Q68L), HA-tagged Rab1 variant mRab1-HA (green). Top row: induced trophozoite (8 h.p.i.) with abnormal distribution of CWP1 (red). Scale bar: 5 µm. Bottom row: cell population at 24 h.p.i. with normal cysts (white arrowheads) and a cyst lacking a CWP1 signal (yellow arrowhead). DIC, differential interference contrast. Scale bar: 10 µm. (B) FACS analysis of cells expressing the wild-type Rab1-HA (purple) or the dominant-negative mRab1-HA (green line) at 24 h.p.i. Surface-exposed CWP1 is detected in unpermeabilized cells. Arrowhead, trophozoites; asterisk, cyst peak. The latter is shifted to the left indicating diminished CWP1 on the surface of many cysts. Cysts lacking CWP1 (Fig. 4A) are probably included in the population of unstained cells (arrowhead). (C) Graphical representation of the stem-loop construct used in mRNA ablation experiments. Red box, CWP1 promoter, gray box, (human Bcl) linker sequence; green box, CWP1 3′UTR. Right panel shows a representative quantitative RT-PCR experiment using mRNA extracted from pooled cells (three biological replicates for each time point). Conditional overexpression of a Rab1-specific stem-loop RNA (left) led to a 60-70% reduction of endogenous Rab1 mRNA levels 4 h.p.i. (PHO/Rab1^{RNA-}) compared with control parasites expressing only the antisense and linker portion of the heterologous RNA (PHO/Rab1). Induction of CWP1 mRNA was equally strong in both cell lines. Bottom left panel shows images of specific phenotype seen in cells after Rab1 mRNA ablation at 24 h.p.i. Morphologically normal cysts in bright-field images show strongly decreased or missing signal after immunostaining of surface exposed CWP1 (yellow arrowhead). White arrowhead indicates a normal cyst. Scale bar: 10 µm.

observed in these cells (Fig. 4C; supplementary material Fig. S2C), albeit with similar low frequency, supporting the notion that this sporadic but consistent phenotype (~1-3% of all cysts) was indeed specific.

Ablation of Arf1 function selectively inhibits cyst wall formation

The small GTPase Arf1 functions as the key regulator of COPI and clathrin membrane coats at Golgi membranes (D'Souza-Schorey and Chavrier, 2006). Transient association of COPI and clathrin coats with early and mature ESVs, respectively, has been demonstrated previously (Marti et al., 2003b). The conserved *Giardia* Arf1 (supplementary material Fig. S1) localizes to punctate structures below the lateral and dorsal plasma membrane, by confocal microscopy, suggestive of an association with the endosomal-lysosomal peripheral vesicle (PV) system (Fig. 5), as observed for giardial clathrin (Gaechter et al., 2008). Arf1 recruitment to ESV membranes in encysting cells is evident at 7-14 h.p.i., which is consistent with the previously observed recruitment of β′-COP and the sensitivity of ESVs to brefeldin A (Hehl et al., 2000; Lujan et al., 1995a).

An essential function of Arf1 in organelle maturation is a central prediction of the Golgi model for ESVs. We tested this using a functional knock-down approach, i.e., conditional expression of a dominant-negative mutant Arf1 (mArf1, Q71L) variant in encysting cells. By initial light microscopy examination, encystation appeared unimpaired in transgenic cells (Fig. 6). Surprisingly, fluorescence microscopy analysis showed that virtually all cyst forms that were positive for mArf1-HA lacked surface-exposed CWP1 (Fig. 6B). However, ESV development appeared normal, which suggested that ER export and partitioning of CWM into ESVs was unaffected (see also supplementary material Fig. S4B).

Controls expressing wild-type Arf1-HA showed complete secretion of CWM and a normal cyst wall (Fig. 6A), whereas cyst forms from the cell line expressing mArf1-HA contained only non-secreted CWP1 in ESV-like organelles (Fig. 6B; supplementary material Fig. S4). Together, this suggests a block in the maturation phase of ESVs, which interferes with their ability to secrete the CWM. Electron microscopy analysis of ultra-thin sections from cysts fixed at 24 h.p.i. confirmed the absence of secreted CWM on these 'naked cysts', despite apparent completion of all other morphological transformations. Quantitative analysis showed a tight encystation phenotype in transgenic cells (Fig. 6C). We also tested whether mutant cysts had lost their resistance to water: a hallmark of bona fide cysts. The 'naked cysts' were completely unprotected and lysed in water, similarly to

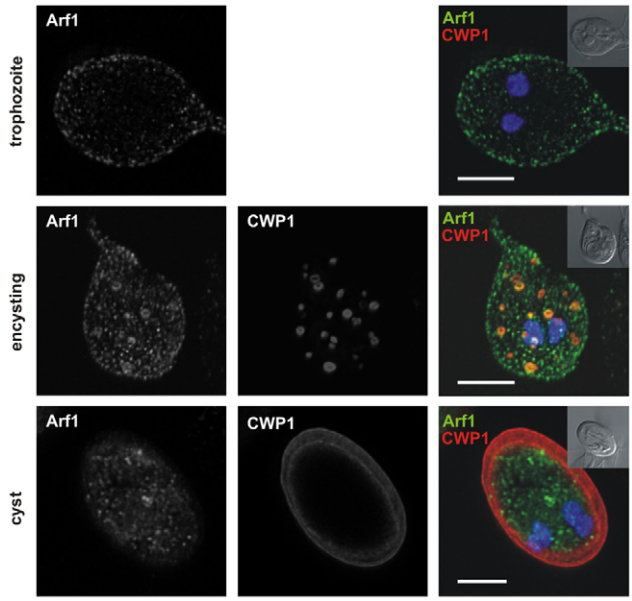

Fig. 5. Subcellular localization of giardial Arf1. Developmentally regulated localization of Arf1 in trophozoites and cysts by confocal microscopy using specific antibodies against recombinant ARF1. In encysting cells at 7-14 h.p.i. Arf1 is clearly recruited to ESVs (middle row). The distribution in uninduced trophozoites is punctate and preferentially near the dorsal plasma membrane (top row), a pattern consistent with labelling of peripheral vesicles similar to the localization of the giardial clathrin heavy chain (Gaechter et al., 2008). Maximum projections are shown. Insets show DIC image. Scale bars: 5 µm.

the previously sensitive trophozoites (supplementary material Fig. S3). Thus, a functional knockdown of Arf1 appeared to decouple cell cycle progression and morphological changes from matrix secretion, suggesting that these coordinated mechanisms are controlled independently during late phase encystation.

ESVs develop into a connected post-ER network of organelles

Functional analysis indicated that ESV neogenesis – formation of morphologically distinct post-ER organelles (Lujan et al., 1995a; Reiner et al., 2001; Stefanic et al., 2006) – is directed by a universally conserved machinery that is also responsible for the genesis of pre-Golgi intermediates and *cis*-Golgi cisternae in higher eukaryotes (reviewed by Stephens and Pepperkok, 2001). ER export of CWM and partitioning into ESVs appears to be completed by 5-8 h.p.i. in our in vitro system; however, ESVs only become secretion-competent just before cyst wall formation at 20-24 h.p.i. A plausible explanation of ESV formation and maturation is the 'pulsed cisternal progression model', where the synchronous maturation of ESVs and cargo is analogous to the *cis* to *trans* maturation of Golgi cisternae (Marti and Hehl, 2003). Because the CWM is secreted quantitatively in only a few minutes, and polymerizes rapidly, key cargo maturation processes (DuBois et al., 2008; Touz et al., 2002) are probably synchronized between the ~30 ESVs in an encysting cell. An elegant solution would be a mechanism allowing direct exchange of CWM between ESVs.

To test whether ESVs are able to exchange CWM during the maturation phase (8-20 h.p.i.), we developed protocols for live-cell imaging by confocal microscopy using a CWP1::GFP reporter shown previously to be exported via ESVs and incorporated into the cyst wall (Hehl et al., 2000). At 10-12 h.p.i. the reporter was typically sequestered in fully formed ESVs (Figs 7 and 8). Transgenic cells were unimpaired in their ability to attach to glass surfaces, which allowed observation in sealed chambers for up to 1 hour. Fluorescence recovery of photobleaching (FRAP) analysis was used to quantify cargo dynamics. Photobleaching of individual organelles resulted in rapid signal recovery, typically reaching a maximum less than 90 seconds after the start of the bleaching cycle (Fig. 7A,B). This was surprising given the highly condensed appearance of the CWM in IFA and EM images. After bleaching of all ESVs in one hemisphere, fluorescence in the target region recovered with similar kinetics (Fig. 7C,D). Quantitative analysis indicated that this recovery was at the expense of the signal in the unbleached half, and the total fluorescence level after 90 seconds stabilized on a significantly lower level. No fluorescence recovery was measured if cells were briefly treated with 1% formaldehyde (data not shown) or if all ESVs in a cell were bleached (Fig. 7E,F). CWP1::GFP was quantitatively secreted with the CWM, and became evenly distributed and completely immobilized (Fig. 7G,H), consistent with polymerization. Taken together the data provide direct evidence for a fully linked ESV organelle network in which the fluid CWM circulate freely. Whether this applies also to CWP2 and CWP3 remains to be determined. An interesting observation was that fluorescence in bleached ESVs never quite recovered to extrapolated levels (see Fig. 7B, asterisk), suggesting that a minor proportion of the reporter was immobilized in ESVs, perhaps forming a condensed core.

Dynamic tubular structures provide the physical link for cargo exchange between ESV compartments

The FRAP experiments suggested that ESVs were immobile at least for the few minutes that these cells were observed. Thus, based on the rapidity of fluorescence recovery, we hypothesized that cargo exchange between ESVs was mediated by permanent or dynamic tubular connections, rather than small COPI- or COPII-coated transport intermediates (Antonny et al., 2001).

To test this, we recorded ESV organelle dynamics for a longer time using time-lapse analysis of attached transgenic cells. Because an ESV-specific membrane marker was not available, the CWP::GFP chimera was used as a luminal reporter for cargo in encysting transgenic cells at 12 h.p.i. The imaging protocol was designed to generate ~1-µm-thick optical sections and to minimize phototoxicity and bleaching. An image series of 100 frames at 21-second intervals (~34 minutes total) showed that ESVs remained stationary in the cell. The movie data also indicated that the organelles undergo considerable change in shape (Fig. 8A,B; supplementary material Movies 1 and 2). However, the resolution of the raw images was insufficient to determine this clearly. To extract the ESV organelle boundaries from background noise, we applied an isosurface function with a fixed threshold value representing organelle boundaries to all frames. This resulted in unbiased models of fluorescence intensity distribution in the time-lapse series (Fig. 8A-C; supplementary material Movie 3) revealing a highly dynamic compartment morphology. Interestingly, the image series suggested the formation and resorption of long tubular structures which dynamically connected ESVs even across cell hemispheres. Previous electron microscopic studies of thin sections suggested a close proximity and even occasional continuity of ER membranes with ESVs (Lanfredi-Rangel et al., 2003). To visualize potential tubular connections between mature ESVs in chemically fixed cells, we used maximum resolution confocal fluorescence microscopy, image deconvolution, and 3D reconstruction. In these

snapshots of cells at >12 h.p.i. labeled with an anti-CWP1 monoclonal antibody, connecting 'channels' as well as blind tubular extensions originating from ESVs were detected (Fig. 8D,E). Interestingly, although difficult to resolve, co-labeling with the ER membrane protein disulfide isomerase 2 (PDI2) showed a clear overlap of signals in some tubular structures (Fig. 8D), raising the possibility that ER membranes might be involved in forming tubular connections between ESVs.

Discussion

Secretory transport requires complex machinery and dynamic compartment organization with the Golgi complex at the center, making study of this system difficult. To address relatively simple questions, for example how cargo moves from the ER through the stack, investigators induce single synchronized traffic waves in mammalian cells (Trucco et al., 2004). Unicellular model organisms, such as *Giardia*, whose trafficking machineries are basic and/or have undergone massive reductive evolution, allow investigation of these fundamental functions in a highly simplified context. Reduction and loss of complexity does not necessarily result in direct reversal of the processes that lead to increasing complexity during eukaryotic evolution. Yet, minimal systems and machineries can extend our view beyond the horizon of the last common eukaryotic ancestor (LCEA) (Dacks and Field, 2007). More importantly, functional analysis of membrane transport systems in *Giardia*, which have a considerably lower complexity than those of the predicted LCEA, could reveal key information about the mechanisms of reductive evolution as a consequence of adopting a parasitic lifestyle. One significant characteristic of the giardial secretory system is the absence of a steady-state Golgi organelle with definable, classically stacked cisternae and faces (Marti et al., 2003a; Elias et al., 2008), which leaves ESVs as the only candidate compartments with a role in post-translational maturation of secretory cargo (Marti and Hehl, 2003). The developmentally regulated genesis of ESVs which are best defined as delay compartments for maturation of a very limited set of pre-sorted secretory material (i.e. CWM) is unique in a highly polarized cell. However, the hypothesis that ESVs are developmentally regulated Golgi analogs that are generated by neogenesis in encysting cells, is difficult to test in the absence of conventional Golgi markers (e.g. GM130, galactosyl transferases or the *trans*-Golgi network marker Rab6) and familiar morphological landmarks. In the present study we therefore focused on predicted functional and structural similarities rather than differences between ESVs in *Giardia* and the Golgi in higher eukaryotes.

ESVs of encysting trophozoites potentially arise by de novo biogenesis, although some form of template structure cannot be excluded. In many higher eukaryotes, Golgi neogenesis is observed after mitotic disassembly and partitioning of Golgi remnants to the daughter cells. However, this mechanism is not universally conserved. The single Golgi stack in the protozoan *Toxoplasma gondii* does not require disassembly (Pelletier et al., 2002), and the Golgi in the forming daughter cell of dividing trypanosomes arises by neogenesis, although it does require some material from the existing Golgi (He et al., 2004). Giardial ESVs are the only known functional Golgi analogs established by de novo biogenesis during stage-differentiation. This is significant in the context of the general simplification and reduction, and/or putative primary basal features in *Giardia*.

In higher eukaryotes the small GTPases Sar1, Rab1 and Arf1 act as molecular switches in this sequence at specific steps during ER export and Golgi transport of secreted proteins (Altan-Bonnet et al., 2004; Aridor et al., 1995). In this study, we interfered with these functions in encysting *Giardia* and elicited distinct phenotypes ranging from failure to progress into stage conversion (Sar1) to a block of cyst wall material secretion (Arf1). Technically, we relied on the conditional expression of dominant-negative mutant variants of the GTPases, which have been used in many different systems to give rise to nonfunctional proteins of the Ras superfamily and block specific functions (Aridor et al., 1995; Dascher and Balch, 1994; Kuge et al., 1994; Lanoix et al., 1999; Memon, 2004; Tisdale et

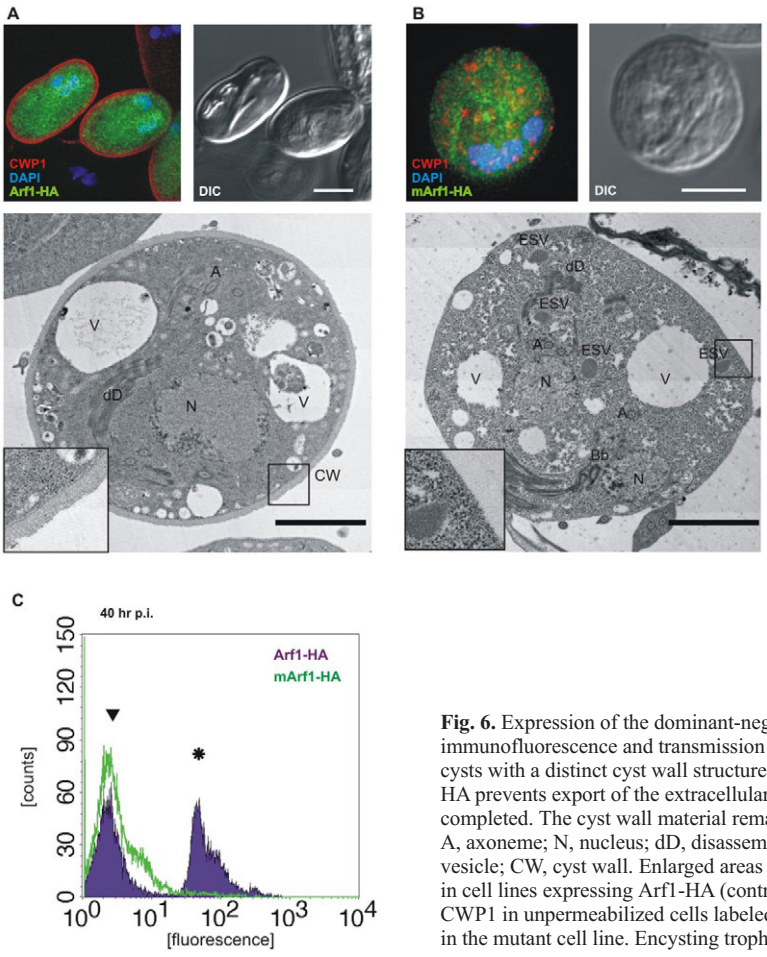

Fig. 6. Expression of the dominant-negative mArf1-HA prevents cyst wall formation. Confocal immunofluorescence and transmission electron microscopic characterization of transgenic cysts. (A) Normal cysts with a distinct cyst wall structure in cells overexpressing wild-type Arf1-HA. (B) Expression of mArf1-HA prevents export of the extracellular matrix, but karyokinesis and morphological transformations are completed. The cyst wall material remains inside. Scale bars: 5 µm (IFA) and 2 µm (tEM). V, vacuole; A, axoneme; N, nucleus; dD, disassembled ventral disk fragment; Bb, basal body; ESV, encystation-specific vesicle; CW, cyst wall. Enlarged areas of the cell surface are shown as insets. (C) Quantification of cyst yields in cell lines expressing Arf1-HA (control) or mArf1-HA at 40 h.p.i. (endpoint). FACS scan of surface-exposed CWP1 in unpermeabilized cells labeled with anti-CWP1. Note the complete absence of the cyst peak (asterisk) in the mutant cell line. Encysting trophozoites (arrowhead).

al., 1992). The well-established conditional expression system controlled by the CWP1 promoter combined with chromosomal integration guaranteed tight repression of these variants in proliferating, and very strong induction in differentiating trophozoites (Hehl et al., 2000). Although interference with functions during early encystation is difficult, this drawback is offset by the tight regulation and the possibility to express lethal genes (Gaechter et al., 2008). By contrast, the only alternative system based on tetracycline-controlled regulation needs 48 hours to produce meaningful amounts of recombinant protein (Sun and Tai, 2000). Post-translational modification (e.g. prenylation) (Calero et al., 2003; Donaldson et al., 2005) required for full functionality of dominant-negative GTPases might delay the inhibitory effect of mutant proteins. For example, by analogy with higher eukaryotes, Sar1 and Rab1 are both expected to be instrumental for early events in ER-to-Golgi transport (Bonifacino and Glick, 2004). However, only interference with Sar1, which does not require post-translational processing, abolished stage-differentiation in most cells, and inhibited ESV formation. This phenotype is consistent with studies using dominant-negative Sar1 in other systems (Aridor et al., 1995; Kuge et al., 1994), and our previous work showing that the giardial cyst wall material is sorted away from constitutively secreted cargo and partitioned into ESVs after its synthesis the ER (Marti et al., 2003a). We detected a sporadic but consistent phenotype of cyst forms that lack CWP on the surface and in internal compartments as a result of mRab1-HA expression or Rab1 mRNA ablation. A possible explanation could be that the phenotype is found only in trophozoites, which respond more slowly to induction. Although distinct developmental stages can be identified easily during encystation, the process has considerable variability. The mRNA ablation approach generally takes longer to produce an effect than expression of a dominant-negative variant. Yet, the requirement for post-translational processing of newly synthesized Rab1 by prenylation probably delays the availability of significant amounts of functional protein. Taken together, this leads to the hypothesis that interference with Rab1 function soon after induction might produce a similarly uniform phenotype as observed for the other GTPases tested here. However, this requires development of a strong and tightly regulated inducible system that is independent of encystation.

Brefeldin A causes the mammalian Golgi to disassemble (Peyroche et al., 1999) and leads to pronounced dispersion of ESVs and blocks cyst formation in *Giardia* (Hehl et al., 2000; Lujan et al., 1995a), suggesting a key role for Arf1 in ESV formation and maintenance. Sorting of CWM into ESVs and organelle morphology were not visibly affected in cells expressing mArf1 variants, but CWM secretion was blocked in almost all encysting cells. This is consistent with a role for giardial Arf1 in essential transport steps between completed establishment of ESVs at 5-8 h.p.i. and exocytosis of CWM at 20-24 h.p.i. Taken together with recruitment of COPI components and the genetic conservation of many other basic components of the trafficking machinery (Marti

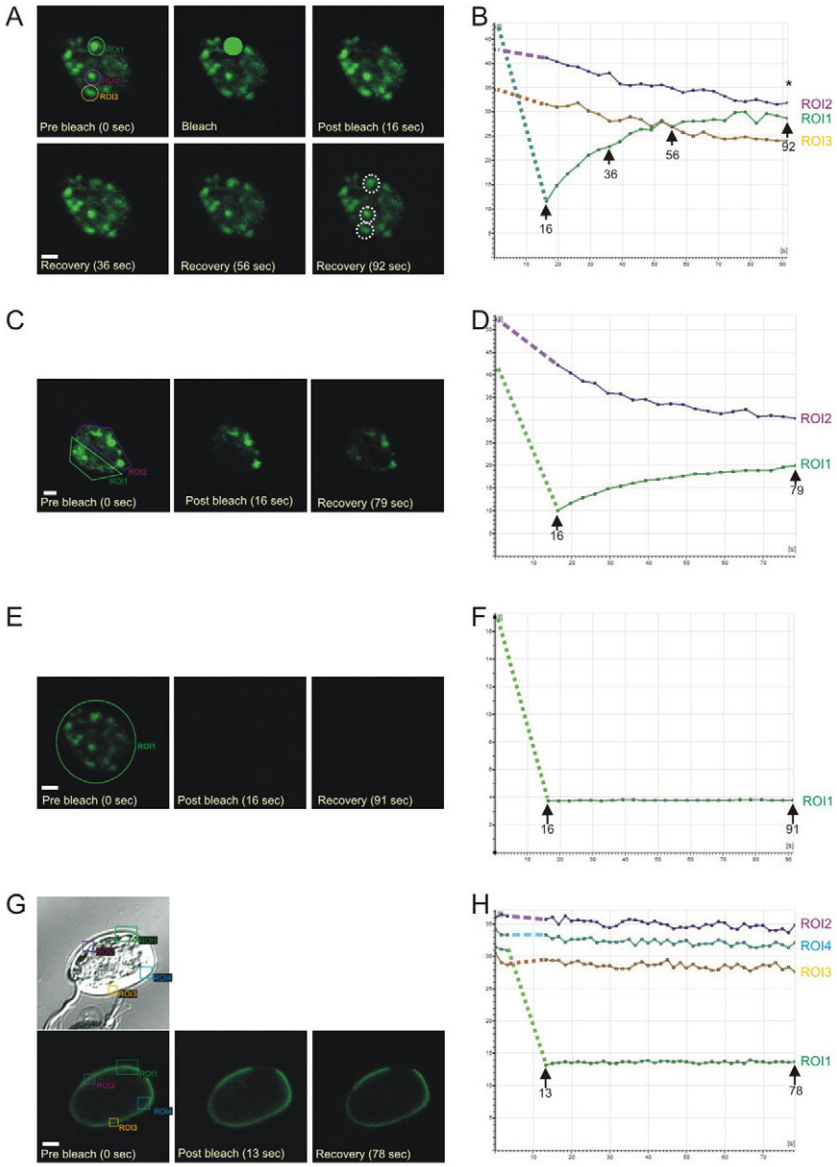

Fig. 7. Fluorescence recovery after photobleaching analysis reveals ESV cargo motility. (A) Single optical sections in a central area of the cell. ESV cargo represented by the CWP::GFP reporter was sequestered almost completely in ESVs with minimal signal observed in surrounding ER structures in a representative attached cell at 12 h.p.i. Region of interest (ROI)1: photobleached organelle. ROI2 and ROI3: control region to assess overall bleaching throughout the experiment (~30%). (B) Quantitative analysis. The signal in ROI1 reaches a plateau at ~75 seconds from the start of the bleaching cycle. Note that the maximal level of recovery in ROI1 is lower than expected based on general bleaching during the imaging process alone (asterisk). Units of fluorescence (*y*-axis) and time in seconds (*x*-axis) are indicated. Time points at which images shown in panels C-F were taken are indicated by arrows. (C,D) Cargo is motile in an ESV network. Photobleaching of all ESVs in one hemisphere (ROI1) of the cell. Recovery of fluorescence in ROI leads to concomitant loss of signal in ROI2. (E,F) No recovery is measured after photobleaching of the entire CWP::GFP pool in ESVs. (G,H) No recovery of fluorescence in a bleached area of the cyst wall (ROI1) is consistent with rapid polymerization of the secreted cyst wall material containing the CWP1::GFP reporter. Scale bars: 2 µm.

et al., 2003b; Morrison et al., 2007), the data support the idea that ESVs develop into analogs of Golgi cisternae.

The requirements and the sequence of events for ESV genesis are remarkably similar to that of early secretory transport leading to *cis*-Golgi formation (Stephens et al., 2000), despite fundamental differences in compartment morphology. However, the exact time when nascent ESVs decouple from ER-exit sites [where sorting of CWM occurs (Marti et al., 2003b)] and assume Golgi characteristics is difficult to pinpoint. Punctate structures that are strongly enriched in Sec31 are observed in close proximity to ESVs (Fig. 2B) up to 8 h.p.i. In the second phase of encystations, this marker disassociates from ESVs together with Rab1 and is replaced with Arf1, β′-COP (Marti et al., 2003b), and later also clathrin (Gaechter et al., 2008). Even though this is consistent with emergence of ESVs from ER-exit sites and development to bona fide Golgi-like post-ER compartments, the physical separation of the ER and ESVs is not clear. EM data (Lanfredi-Rangel et al., 2003) suggest that continuities even with mature ESVs are a possibility. However, resident proteins of both compartments, such as PDIs, BiP and CWPs, are clearly separated. In support of distinct compartment identities, BiP has been shown to cycle through ESVs, from where it is retrieved by the C-terminal ER-retention-signal KDEL (Stefanic et al., 2006). If this signal is mutated, a recombinant BiP distributes evenly in ER and ESVs.

The boundaries between the ER and mature ESVs remain imprecise because of the extensive and dynamic linkage of ESVs by tubular membrane connections. We propose that exchange of cargo and membrane material ensures simultaneous maturation of ESVs. The CWM is sequestered in ~30 fully developed ESVs for the last 10-14 hours of encystation and undergoes post-translational maturation before being exocytosed in a few minutes and polymerizing on the surface. This requires coordination between organelles, presumably by allowing cargo to distribute inside an ESV network structure to ensure that all secreted CWM is fully mature and functional. The nature of these transport intermediates is less clear, however, but probably involves large tubulovesicular rather than small spherical membrane carriers (e.g. classical COPI vesicles). One scenario for the genesis of these connections is that they are formed from ESV membrane material analogous to mechanisms for Golgi ribbon formation (Trucco et al., 2004; Griffiths, 2000; Beznoussenko et al., 2007). Formation of such higher-order compartment structures in cells of mammals, plants and insects is thought to lead to more uniform distribution of processing enzymes. However, key factors involved in Golgi ribbon formation, such as GM130 or GRASPs (Feinstein and Linstedt, 2007; Feinstein and Linstedt, 2008) cannot be identified in the *Giardia* Genome Database, suggesting that this machinery is not universally conserved in eukaryotes. An alternative scenario is that maturing ESVs recruit tubular ER structures to establish inter-organelle connections or maintain connections with specialized subdomains of the ER for the purpose of exchanging cargo. This scenario is supported by co-labeling of CWP in tubular structures with the ER marker. Distinguishing between these two possibilities to explain this novel phenomenon is directly linked to the nature of ESVs as putative Golgi analogs. Despite accumulating evidence for this idea, the current data does not allow us to determine with certainty whether ESVs should be considered specialized ER subdomains, ER-Golgi intermediate compartments, bona fide Golgi cisternae, or, more likely, all of the above at different stages of development. Addressing this question requires a thorough analysis of the discrete ER-exit sites (ERES) in *Giardia* and their role in CWM export. Indeed, preliminary experiments using epitope-tagged Sec23 indicate that ESVs are nucleated by export of CWM at closely associated ERES but become rapidly independent as they accumulate cargo (A.B.H. and Christian Konrad, Institute of Parasitology, University of Zurich, Switzerland, unpublished). This constitutes strong evidence for the Golgi character of ESVs but does not exclude the possibility of an intimate association with some ER domains that persist throughout the encystation process.

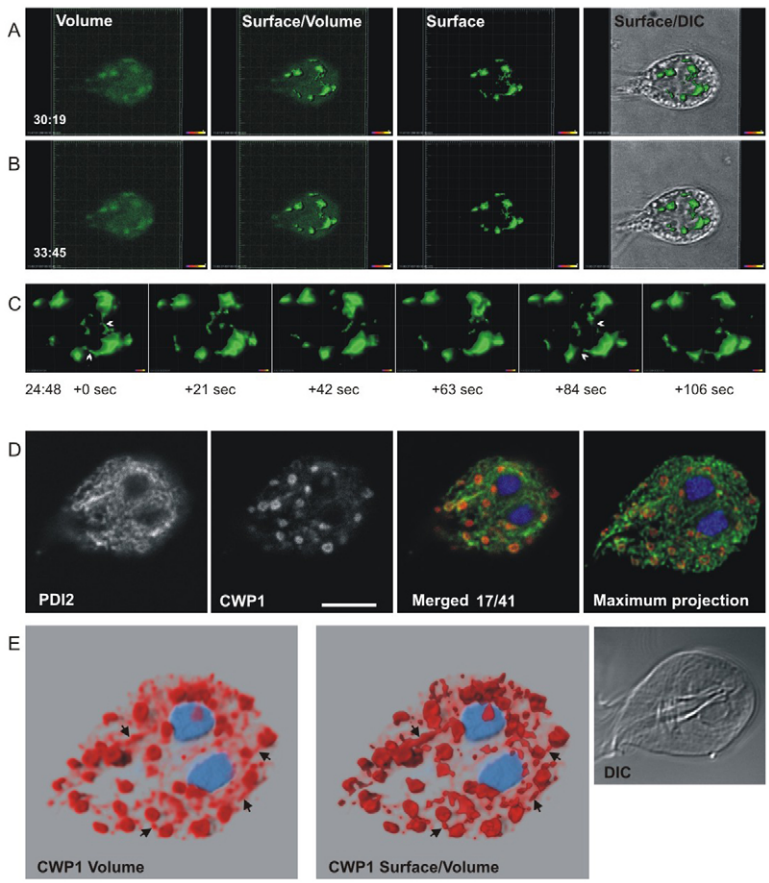

Fig. 8. Dynamic tubular connections between ESVs contain cargo material. (A,B) Time-lapse series of transgenic cells: rows show single frames of the 100 frame sequence (34 minutes total). Isosurface rendering of raw images (left) was used to model organelle boundaries. Differential interference contrast (DIC) images show morphological features of the cell. See also supplementary material Movies 1-3. (C) Isosurface model of six frames documenting organelle dynamics during 106 seconds starting at 24 minutes 48 seconds. Arrowheads indicate dynamic connections between ESV organelles. (D) Confocal microscopy of the secretory system in a representative chemically fixed cell at 12 h.p.i. Antibodies against protein disulfide isomerase 2 (PDI2) and CWP1 represent the ER and ESV compartments, respectively. A single optical section (section 17 of 41), and a maximum projection of the deconvolved image stack is shown. (E) Volume (left image) and surface rendering (middle image) of the CWP1 signal in the entire image stack. Arrows indicate tubular structures with sufficient signal strength to appear in the surface model. A DIC image of the cell is shown on the right. Nuclear DNA is stained blue with DAPI. Scale bars: 5 μm.

Materials and Methods

Culture media, cells and transfection

Trophozoites of the *Giardia lamblia* strain WBC6 (ATCC catalog number 50803) were grown in 11 ml culture tubes (Nunc, Roskilde, Denmark) containing TYI-S-33 medium supplemented with 10% adult bovine serum and bovine bile according to standard protocols (Hehl et al., 2000). Plasmid vector DNA was linearized using *Swa*I restriction enzyme and 15 μg digested plasmid DNA was electroporated (350 V, 960 μF, 800 Ω) into trophozoites. Linearized plasmid targets the *Giardia lamblia* triose phosphate isomerase (*TPI*) locus (see below) and integration occurs by homologous recombination under selective pressure with the antibiotic puromycin (Sigma) (Jimenez-Garcia et al., 2008).

Parasites were harvested by chilling the culture tubes on ice for 30 minutes to detach adherent cells, and collected by centrifugation at 1000 ***g*** for 10 minutes. Cells were then resuspended in phosphate-buffered saline (PBS) and counted using the improved Neubauer chamber.

Encystation was induced using the two-step method as described previously (Hehl et al., 2000), by cultivating the cells for 44 hours in medium without bile and subsequently in medium with higher pH and porcine bile.

Expression vector construction

All constructs were based on the expression cassette C1-CWP for inducible expression under the control of the CWP1 promoter (Hehl et al., 2000). Giardial Sar1, Arf1 and Rab1 (GenBank accession numbers are listed in supplementary material Table S2) coding regions were amplified by PCR from genomic DNA and cloned into *Nsi*I and *Pac*I sites of the C1-CWP vector containing the hemagglutinin (HA) epitope tag upstream of the *Nsi*I restriction site, as described previously (Stefanic et al., 2006), or in the identical vector variant without an HA tag. Single amino acid mutations in the GTPase domains of Sar1, Arf1 and Rab1 were introduced by site-directed mutagenesis (supplementary material Table S1, mutated codon is shown in bold letters). The chimeric CWP1::GFP reporter used in the study was described previously (Hehl et al., 2000). For this study, it was subcloned into the pPacV-Integ vector for chromosomal integration (supplementary material Fig. S5) (Jimenez-Garcia et al., 2008). The pPacV-Integ expression vector for stable integration was designed as a double cassette with a constant part containing a bacterial puromycin-resistance gene under the control of the *Giardia* glutamate dehydrogenase gene promoter (*GDH*, GenBank accession number M84604). Genes of interest including promoter sequences were cloned into adjacent *Xba*I and *Pac*I restriction sites upstream of *CWP1* 3′ flanking sequences. The puromycin-resistance gene and the expression cassette were in head-to-head orientation. Targeting to the intergenic region adjacent to the triose phosphate isomerase gene locus (*TPI*, Giardia DB GL50803-93938) is facilitated by flanking regions (GL50803-17200 and GL50803-93938, respectively), amplified from genomic DNA of *Giardia lamblia* strain WBC6 using corresponding primer pairs (see supplementary material Table S1), digested and ligated between the *Cla*I and *Xba*I restriction sites of the pBluescript KS(–) vector (Stratagene, La Jolla, CA).

Antibody production

The open reading frames of Sar1 (aa 7-189), Rab1 (aa 1-212) and Arf1 (aa 1-191) were PCR-amplified, digested with *Eco*RI, *Pst*I (Sar1), *Xba*I, *Hin*dIII (Arf1) or *Bam*HI, *Sal*I (Rab1), and inserted downstream of the maltose-binding protein (MBP) in plasmid vector pMal-2Cx (New England Biolabs, MA). The fusion proteins were produced in transgenic *Escherichia coli* by induction with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 hours at 37°C. MBP fusions were isolated from bacterial lysates by affinity purification on amylose resin according to the manufacturer's protocol (New England Biolabs, MA), dialyzed and lyophilized. NMRI mice were immunized intraperitoneally with 100 μg of fusion protein emulsified with RIBI adjuvant (Corixa, Hamilton, MT) on days 0, 15, 30 and 50. Blood from the tail vein was collected before the initial immunization and after the second and third boost. The serum fraction was assayed for the presence of specific antibodies by western blot (supplementary material Fig. S6) using separated total *Giardia* trophozoite lysate and by IFA (immunofluorescence assay). Only sera reacting with a single, specific band of the expected size were used for assays.

Protein analysis

Giardia parasites were harvested for gel electrophoresis by chilling culture tubes in ice and centrifugation at 1000 ***g***. The cell pellet was washed once in ice-cold phosphate-buffered saline (PBS) and counted in a Neubauer chamber. The cell pellet was dissolved in SDS sample buffer to obtain 2×10^5 cells in 50 μl and boiled for 3 minutes. Dithiothreitol (DTT) was added to 7.75 μg/ml before boiling. SDS-PAGE on 12% polyacrylamide gels and transfer to nitrocellulose membranes was done according to standard techniques. Nitrocellulose membranes were blocked in 5% dry milk, 0.05% TWEEN-20 in PBS and incubated with polyclonal antibodies against Sar1 (1:400), Sec31 (1:200), Rab1 (1:300) and Arf1 (1:500) in blocking solution. Bound antibodies were detected with horseradish-peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA), respectively, and developed using Western Lightning Chemiluminescence Reagent (PerkinElmer Life Sciences, Boston, MA). Data collection was done in a MultiImage Light Cabinet with AlphaEaseFC software (Alpha Innotech, San Leonardo, CA) using the appropriate settings.

Immunofluorescence microscopy

Cells were harvested by cooling and centrifugation at 1000 ***g*** for 10 minutes. Fixation and preparation for fluorescence microscopy was done as described previously (Marti et al., 2003a). Briefly, cells were washed with cold PBS and fixed with 3% formaldehyde in PBS for 40 minutes at 20°C, followed by a 5 minute incubation with 0.1 M glycine in PBS. Cells were permeabilized with 0.2% Triton X-100 in PBS for 20 minutes at room temperature and blocked overnight in 2% BSA in PBS. Incubation of all antibodies was done in 2% BSA, 0.2% Triton X-100 in PBS. Titrated polyclonal antibodies raised against giardial Sar1, Sec31, Rab1 and Arf1 were incubated for 1 hour at room temperature. Alexa Fluor 488-conjugated secondary antibodies were used as 1:200 dilutions and incubated under same conditions (Molecular Probes, Eugene, OR).

Mouse monoclonal Alexa Fluor 488-conjugated anti-HA (Roche Diagnostics, Manheim, Germany; dilution 1:30) or Cy3-conjugated anti-CWP1 (Waterborne, New Orleans, LA; dilution 1:80) were incubated for 1 hour at 4°C. Washes between incubations were done with 0.5% BSA, 0.05% Triton X-100 in PBS. Labeled cells were embedded with Vectashield (Vector Laboratories, Burlingame, CA) containing the DNA intercalating agent 4′-6-diamidino-2-phenylindole (DAPI) for detection of nuclear DNA. Immunofluorescence analysis was performed on a Leica SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) a glycerol objective (Leica, HCX PL APO CS 63×, 1.3 Corr). Image stacks were collected with a pinhole setting of Airy 1 and twofold oversampling. Image stacks of optical sections were further processed using the Huygens deconvolution software package version 2.7 (Scientific Volume Imaging, Hilversum, The Netherlands). Three-dimensional reconstruction, volume and surface rendering were done with the Imaris software suite (Bitplane, Zurich, Switzerland).

Flow cytometric analysis

For quantification of cyst yield in cells expressing giardial Sar1 and Arf1 mutant variants, parasites were induced to encyst for 20 or 40 hours. Cysts and detached trophozoites were collected from confluent cultures by pouring off the culture medium in another tube and rinsing the culture tube twice with prewarmed PBS (37°C). Cells were pelleted by centrifugation at 1000 ***g*** for 10 minutes and processed following the protocol for immunofluorescence (see above) without a permeabilization step. Cysts were fluorescently labeled for 1 hour at 4°C using monoclonal Cy3-conjugated anti-CWP1 antibody (Waterborne, New Orleans, LA; dilution 1:80). Before FACS analysis, cells were washed twice in PBS. Unlabeled samples were used to determine background fluorescence, and subsequently, fluorescently labeled cysts were analyzed in triplicate on a FACSCalibur flow cytometer (Becton & Dickinson, Basel, Switzerland).

For quantification of the mutant Rab1 phenotype, attaching trophozoites and cysts from a culture at 24 h.p.i. were separated by collecting the culture supernatant. Cysts and were collected by centrifugation at 1000 ***g*** for 10 minutes and washed in PBS. Samples were processed and analyzed as described for Sar1 (see above). All samples were analyzed in parallel by IFA to assess encystation efficiency and quality of separation of cysts from trophozoites.

Water treatment of cysts

Cells were induced to encyst (as described above) for 24 hours. The cysts were harvested as described, resuspended in PBS and counted using bright-field microscopy. One half was incubated in 1 ml water, the other with 1 ml PBS overnight at 4°C and counted again. To verify counting, the cells were then fixed and processed for IFA as described above. Statistical analysis was performed using the Comprehensive Meta Analysis software (Version 2.2.046, Biostat, Englewood, NJ).

Gene silencing by RNA ablation

For Rab1 mRNA ablation, a vector for conditional expression of a stem-loop Rab1 double-stranded RNA under the CWP1 promoter was constructed (Fig. 4C). The Rab1 sequences were amplified from genomic DNA using oligonucleotide primers shown in supplementary material Table S1. The Rab sequences were cloned head to head and separated by 750 bp of the human Bcl gene as a spacer. A control construct containing only the antisense sequence of Rab1 followed by the Bcl-linker sequence was used.

RNA isolation and cDNA synthesis

Total RNA was isolated using the Rneasy Mini Kit Qiagen (Qiagen, Hilden, Germany) including a DNase digest (DNase Kit Qiagen). First-strand cDNA synthesis was performed according to standard protocols using an anchored oligo-dT primer and 50 U SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The reaction was incubated at 45°C for 50 minutes and heat inactivated at 70°C for 15 minutes, chilled on ice and followed by digestion with 10 U RNase H for 20 minutes at 37°C.

Quantitative RT-PCR

The standard curves for the iCycler (Bio-Rad) to calculate the slope of each primer pair was generated with Rab1, CWP1, protein phosphatase 1 (PHO) dilutions in duplicate (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) using the following cycling conditions: 15 minutes 94°C, 15 seconds 94°C, 20 seconds 58°C, 30 seconds 72°C. PHO mRNA is not regulated during differentiation. PCR amplification was done in triplicate for each

time point and primer pair (PHO-s-quant, CWP1-s-quant, Rab1-s-quant, k-adaptor) (see supplementary material Table S1).

Efficiency was calculated using the equation $e=10^{(-1/slope)}$. The ratio of standard (PHO, CWP1) to target (Rab1) (est×nst/etg×ntg, where est, efficiency of the standard; etg, efficiency of the target; nst, cycle number of the standard at threshold; ntg, cycle number of the target at threshold) was calculated using the Gauss error function. Percentage induction was calculated by setting the Rab1 mRNA level at time point 0 hour to 50%. Levels of CWP mRNA at 24 h.p.i. were set to 100%.

Live-cell microscopy and FRAP analysis

For live-cell microscopy, induced cells expressing the CWP1::GFP chimera were harvested at 12 h.p.i. and transferred to 24-well plates at a density of 6×10^6/ml. After incubation on ice for 5-8 hours, oxygenated cells were sealed between microscopy glass slides and warmed to 21°C or 37°C. Under these conditions, the encysting cells were stable and even continued to complete encystation. For FRAP and time-lapse series, images were collected on a Leica SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) using a 63× water immersion objective (Leica, HCX PL APO CS ×63, 1.2 W Corr). The pinhole was set to Airy 2 to increase the thickness of the optical sections to accommodate an entire ESV in the *z*-plane. Quantifiable criteria for cell viability were active attachment to substrate and continuous beating of the ventral and anterolateral flagella pairs. FRAP experiments were performed as described (Gaechter et al., 2008) using the Leica FRAP software module to set bleaching parameters and quantify fluorescence recovery. Movies and surface rendering of raw time-lapse series were generated using the Imaris software suite (Bitplane, Zürich, Switzerland).

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PART IV

Manuscripts

2) Differentiation in encystation in *Giardia lamblia* is determined by a small set of stage-regulated genes

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Manuscript in preparation

This study represents the major part of my PhD work. I started with this project in the second year of my PhD. All ideas, the experimental procedures, the manuscript content and structure were developed and defined by me and my direct supervisor Prof. Adrian B. Hehl.

All experimental and bioinformatical work (except the bioinformatical data processing of the microarray analysis which was done in collaboration with the functional genomics center Zurich (FCGZ) by Dr. H. Rehrauer) as well as all the resulting figures for the manuscript were done by me. This project also required the establishment of the microarray technique in our laboratory. This has been done in collaboration with FGCZ by me and Dr. Cornelia Spycher.

Differentiation in Encysting *Giardia lamblia* is Determined by a Small Set of Stage-regulated Genes

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ABSTRACT

Flagellated trophozoites of the protozoan parasite *Giardia lamblia* undergo a stage-differentiation process of approximately 24 hours to transform to environmentally resistant and infectious cysts. Parasites complete encystation in the small intestine and become encased in an extracellular matrix comprised of three cyst wall proteins (CWPs) and a β (1-3) - GalNAc homopolymer. Differentiation in the host gut is induced by as yet undefined environmental cues and leads to a sharp upregulation or induction of mRNAs coding for CWPs, factors involved in synthesis of the glycan and a transcription factor Myb2. *In vitro*, changes in bile concentrations and sources, pH, and availability of lipid (cholesterol) in the medium are used to induce differentiation in distinct but similarly effective protocols. Several large-scale analyses have been implemented to identify the molecular underpinnings of differentiation as a basis for the characterization of this key process. However, the conditions for induction are not standardized and, more importantly, significant side-effects are expected from each protocol. Here, we present a comparative microarray analysis to determine the complement of *bona fide* encystation-specific genes which are upregulated on the transcriptional level. We show that the transcriptional response to the most widely used encystation protocol results in a bipartite response with surprisingly minor involvement of stress genes. Our results suggest that a majority of genes in each protocol are induced as a side-effect of culture conditions. We also identify a Myb binding sequence as a signature motif in promoters of encystation-specific genes. Taken together, the data suggest coordinated regulation of a small set of encystation-specific genes, which code in the majority for structural components of the cyst wall or factors involved in their synthesis and assembly.

INTRODUCTION

Differentiation of *Giardia lamblia* to cysts is a key step in the simple life cycle of this ubiquitous intestinal parasite. Proliferating trophozoites, the flagellated, motile, and attachment competent stage exit the cell cycle at the G2-M transition [1] and begin to synthesize and export the components of an extracellular matrix, the cyst wall (CW), which eventually completely encloses the parasite and makes it environmentally resistant [2,3]. This process, termed encystation, can be triggered also *in vitro* using changes in medium components or concentrations, e.g. bile, fatty acids (cholesterol) or lactic acid [4-7]. Interestingly, none of these changes alone are very effective in inducing encystation without a concomitant increase in culture medium pH to 7.85. The pH change alone, however, is not sufficient to trigger encystation, thus it is likely that induction of the genetic program which leads to cyst formation requires multiple signals. The condition(s) which initiate encystation *in vivo* are not known, however, but all factors and components, mentioned above, are implicated since their concentrations vary in the small intestine, as does the pH which increases from ~6 in the duodenum to 7.5-8.0 in the distal ileum. Although it is undisputed that differentiation is induced by one or several environmental signals, the mechanism(s) for reception and transduction are not known. Using various small-scale but also large-scale analysis methods [8-13], upregulation of genes coding for the three major structural proteins and enzymes involved in the synthesis of the glycan component of the cyst wall has been documented. Considering how effectively the cyst wall protects the parasite in the environment, the exported CWM appears to have a surprisingly low complexity: The three paralogous cyst wall proteins (CWP1-3) have been identified as the major protein components of the structure [12,14], in addition to a simple β (1-3) - GalNAc homopolymer glycan [15,16], which contributes ~60% of the CWM,

but whose manner of integration with CWPs in this extracellular matrix is unknown. A membrane-anchored, type 1 cysteine-rich protein (HCNCp) which localizes to the endoplasmatic reticulum and the plasma membrane of cysts [17] could function as a possible link between the CW proper and the cell surface. The three CWPs are paralogous members of a protein family and share a high degree of homology [12]. The CWP genes are silent in trophozoites; transcript levels during encystation *in vitro* peak around 7 h post induction (p.i.) and appear to be sharply downregulated after that [18,19]. Inducible expression of a reporter under the control of a CWP1 promoter showed that down-regulation of this mRNA after peak induction was dependent on a short downstream region flanking the CWP1 ORF [19]. Another category of upregulated genes, beside those coding for structural proteins, are factors needed for the synthesis of the cyst wall glycan. Transcription of five enzymes involved in synthesis of UDP-GalNAc from glucose was found to be induced relatively late during encystation [20]. At least one of those enzymes, UDP-N-acetylglucosamine phosphorylase, is also allosterically regulated [21].

Several leads have been developed how expression of CWPs and the enzymes involved in cyst wall biosynthesis may be induced. A Myb2-like protein is one of the few transcription factors identified in Giardia [10,22,23]. Recently, a plant-like WRKY protein was also shown to bind to promoter sequences and influence transcription levels. Both *trans* acting factors have binding sites inside <200 bp upstream of CWP1, CWP2, and several other factors involved in biosynthesis of the cyst wall. While it seems clear that the giardial Myb2 plays a role in regulating transcription of CWPs and itself by binding to short sequence motifs upstream of these genes, the literature does not quite agree on what these motifs are. Random site selection experiments turned up a GTTT(G/T)(G/T) motif and Myb2 was shown to bind to CWP1 and Myb2 promoters [10]. A paper by the Gillin group described a binding motif C(T/A)ACAG in the upstream regions of the Myb, CWP1-3 and G6PI-B genes to which this putative transcription factor binds [23]. Interestingly, three Myb binding sequences (MBS) cloned upstream of a constitutively expressed gene confer only weak stage-specific regulation (2.6 fold), but if cloned upstream of the regulated G6PI-B gene promoter boosts induction >10 fold. This suggests that Myb reinforces transcriptional upregulation but cannot act alone. A binding motif (c/t)TGAC(c/t) for a giardial WRKY protein has been identified in the upstream region of the stage-regulated *wrky*, *cwp1*, *cwp2*, and *myb2* genes, but also in a number of non-regulated genes [24]. The WRKY binding motif appears to act as a positive regulatory element of transcription in vegetative and in differentiating cells. An interesting aspect of encystation-specific promoter function was revealed by a deletion study of the CWP2 upstream sequence. Davis-Hayman and coworkers [18] showed that deletions in a 64 nt region upstream of the CWP2 gene led to expression of a reporter also in proliferating trophozoites, effectively abolishing stage-regulation. This is direct evidence that transcription of the CWP2 gene is silenced in trophozoites and de-repressed upon induction of encystation.

Taken together, the current data indicate that rapid encystation-specific induction of gene expression is conferred on multiple levels which encompass basic transcription activity, enhancement and de-repression, but no single factor or *cis*-acting motif has a dominant role. Thus, without a larger sample set of encystation-specific genes it is uncertain if all upregulated genes can be identified by a simple *in silico* approach. In addition, the lack of a Myb binding site in three of the five induced genes of the UDP-GalNAc synthesis pathway may point to differences in regulatory mechanisms between genes which are induced at early (such as CWPs) of at later stages of encystation [20]. Another key aspect is how induction of encystation *in vitro* is performed. Until now, none of the experimental approaches to identify encystation-specific genes, including those used to generate the large amount of SAGE data posted on the Giardia DB website, include the possibility to differentiate between gene products which are really involved in the encystation process and those which are upregulated unspecifically as a side effect of the particular encystation protocol applied. Analysis of encysting parasites directly from the gut of a host could be a gold standard, but this has not been possible for technical reasons and would also require synchronization of induction which is not feasible *in vivo*.

Here, we test the hypothesis that induction of encystation *in vitro* activates the transcription of a very limited number of genes coding for products that are directly required for differentiation. We predict that transcriptional induction of many other factors should be considered protocol-specific off-target effects which are neither (directly) linked to nor required for encystation. This is an important issue since all available data on encystation-specific gene expression (individual genes and large scale datasets, *e.g.* SAGE and microarray) were generated with only one protocol. To address this issue, we focused our analysis on the first 7 h post induction. During this time the bulk of CWPs is synthesized and exported from the endoplasmic reticulum to specialized organelles, encystation-specific vesicles (ESVs), for maturation. To identify encystation-specific genes we used mRNA from parasites induced with two distinct but equally effective encystation protocols to generate microarray datasets. Comparative analysis of the 7 h p.i. datasets revealed that upregulation of a small core set of genes

is required for differentiation and underscores the importance of the Myb binding sequence as a signature motif of bona-fide differentiation-specific gene promoters.

RESULTS AND DISCUSSION

Induction of encystation *in vitro* triggers a bipartite transcriptional response. Induction levels of CWP mRNA expression mentioned in the literature range from 100 to 300 fold with low background expression in trophozoites [12,14,25]. Considering that there is a "spontaneous" rate of encystation of up to a few percent in normal trophozoite cultures, it is more likely that CWP expression is completely silenced at that developmental stage. Peak mRNA levels of the most abundant proteins exported to the cyst wall (CWP1-3) in induced parasites are detected at ~7 h p.i. [19]. To investigate the transcriptional induction of cargo exported to ESVs we therefore focused our analysis on this initial stage of the differentiation process which is relatively synchronous. RNA was isolated from encysting trophozoites at 45 min, 3 h, and 7 h p.i and used for dual color hybridization to a full genome microarray with uninduced trophozoite RNA as the reference sample. For this time-dependent analysis we used the standard "two-step" method for induction [5,19], henceforth referred to as protocol A. The three datasets containing spots with a signal increase of >2 comprise a total of 49 different genes. Figure 1 shows that the "early" (45 min, 16 hits) and "late" (7 h, 28 hits) datasets do not overlap. The "intermediate" dataset (3 h, 15 hits) is the smallest and has four unique positions, two overlaps with the "early" dataset, and shares 10 positions with the "late" dataset. Together with the signal increase in all but two of these shared positions at 7 h p.i. this indicates that the transcriptional response is divided into an early phase which disappears before 3 h p.i., and a late phase which is sustained through 7 h p.i. Only four genes are unique to the 3 h time point. The mRNAs coding for the CWPs 1-3, an HCNCp, and the transcription factor Myb2 are strongly upregulated after 3 h p.i. In addition, several genes associated with synthesis of the CW glycan [20] are upregulated in the "late" fraction indicating that this dataset includes also genes coding for later steps in CW synthesis. In total, mRNAs from all previously identified regulated genes are included in the combined 3 h and 7 h datasets which validates the approach used here.

Taken together we find a qualitatively significant but quantitatively limited, bipartite transcriptional response to the encystation stimulus in this microarray study. This fits with the idea that encystation requires relatively small changes in expression patterns except for the synthesis of the CW components [26].

Figure1

A

ORF	Description	fold		
7h		7h	3h	45min
8245	Glucosamine-6-phosphate deaminase	5.3		
10552	Hypothetical Protein	3.7		
14626	Oxidoreductase	3.3		
92729	Fatty acid elongase 1	2.8		
88581	Synaptic glycoprotein SC2	2.7		
7388	Hypothetical Protein	2.2		
12082	Hypothetical Protein	2.5		
7134	Hypothetical Protein	2.4		
103785	Hypothetical Protein	2.4		
14759	6-phosphogluconate dehydrogenase	2.3		
16069	phosphoacetylglucosamine mutase	2.3		
21924	kinase, NEK	2.2		
3063	Hypothetical Protein	2.2		
9115	Glucose-6-phosphate isomerase	2.1		
4846	Protein 21.1	2.1		
106496	Hypothetical Protein	2.1		
24412	Protein 21.1	2.1		
102813	Protein 21.1	2.1		
7139	Hypothetical Protein	2.0		
5435	Cyst wall protein 2	24.7	15.9	
5638	Cyst wall protein 1	19.6	12.4	
9046	Sugar transport family protein	9.1	6.1	
32657	Hypothetical Protein	8.5	2.4	
8722	Myb 1-like protein	4.2	3.4	
2421	Cyst wall protein 3	4.0	2.2	
137701	kinase, NEK	2.8	2.1	
112432	high cysteine membrane protein Group 5	2.2	2.4	
88814	Protein kinase	2.2	2.0	
7260	Aldose reductase	2.1	2.2	
3h				
3643	70 kDa peptidylprolyl isomerase		2.9	
98054	Heat shock protein HSP 90-alpha		2.7	
88765	Hsp70 (cytosolic)		2.4	
10429	Wos2 protein		2.0	
10570	FKBP-type peptidyl-prolyl cis-trans isomerase		2.2	2.4
17012	Hypothetical Protein		2.0	2.7
45min				
9068	Hypothetical Protein			2.8
17012	Hypothetical Protein			2.5
3595	Endonuclease III			2.5
16519	AstB/chuR-related protein			2.4
17587	EC#6.3.4.2			2.4
16602	Hypothetical Protein			2.3
10341	Hypothetical Protein			2.3

B

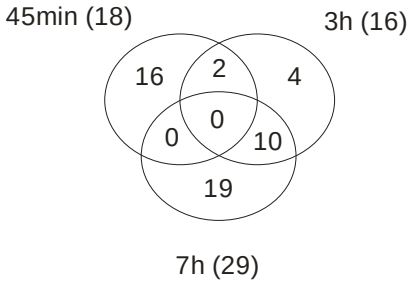


Figure 1 Whole genome microarray analysis of encystation induced by Protocol A. A) List of statistically upregulated genes (cut-off > 2 fold signal increase) at 45 min., 3 h, and 7 h post induction. B) Graphical representation (Venn diagram) of overlaps in the three datasets. Note that no gene is present in all three datasets.

Two different protocols result in highly comparable induction of encystation

Since the 7 h dataset generated with cells encysted with protocol A contained all previously identified regulated genes, we decided to use this time point for a comparative analysis of encystation protocols with the aim of filtering out off-target effects. Culture of trophozoites in cholesterol (lipid) – free medium and elevated pH (protocol B) is an efficient (albeit more expensive) method to induce encystation. As a first step we tested if the kinetics of induction elicited by protocols A and B were comparable. As criteria we used expression of CWP1 protein, ESV formation and appearance of cysts. FACS analysis of chemically fixed and detergent-permeabilized cells labeled with anti-CWP1 mAb showed that the kinetics of CWP1 appearance during the first 8 h p.i. was very similar with both protocols (Figure 2A). Immunofluorescence analysis at 4 h and 8 h p.i. confirmed formation of ESVs. Quantitation of encysting cells and cysts at 7 h and 24 h p.i. revealed a higher cyst output with protocol B at 24 h p.i. In these tests we also confirmed the importance of an elevated pH (7.85) for induction using protocol B (Figure 2B). Conversely, increase of the medium pH alone is not sufficient for induction.

It is likely that *in vivo*, elevated pH in combination with another signal involving lipids is required to induce encystation, indicating that differentiation is only triggered at specific locations in the small intestine. The combination of two signals suggests that temporal as well as spatial criteria are important. It is tempting to speculate that the latter may provide a way to segregate rapidly proliferating trophozoites from differentiating parasites. Since conditions change significantly between the duodenum and the distal ileum, an interesting question in this respect is how long inducing conditions have to persist for the cells to complete encystation. Preliminary *in vitro* data suggest that regardless of the protocol used, this time is >7 h but <20 h p.i.. Parasites which are brought back to normal culture conditions before 7 h p.i. do not progress to cysts but become often severely damaged (data not shown). This indicates that inducing conditions do not trigger an all-or-nothing response but parasites need sustained stimulation to complete the encystation process.

Fig2

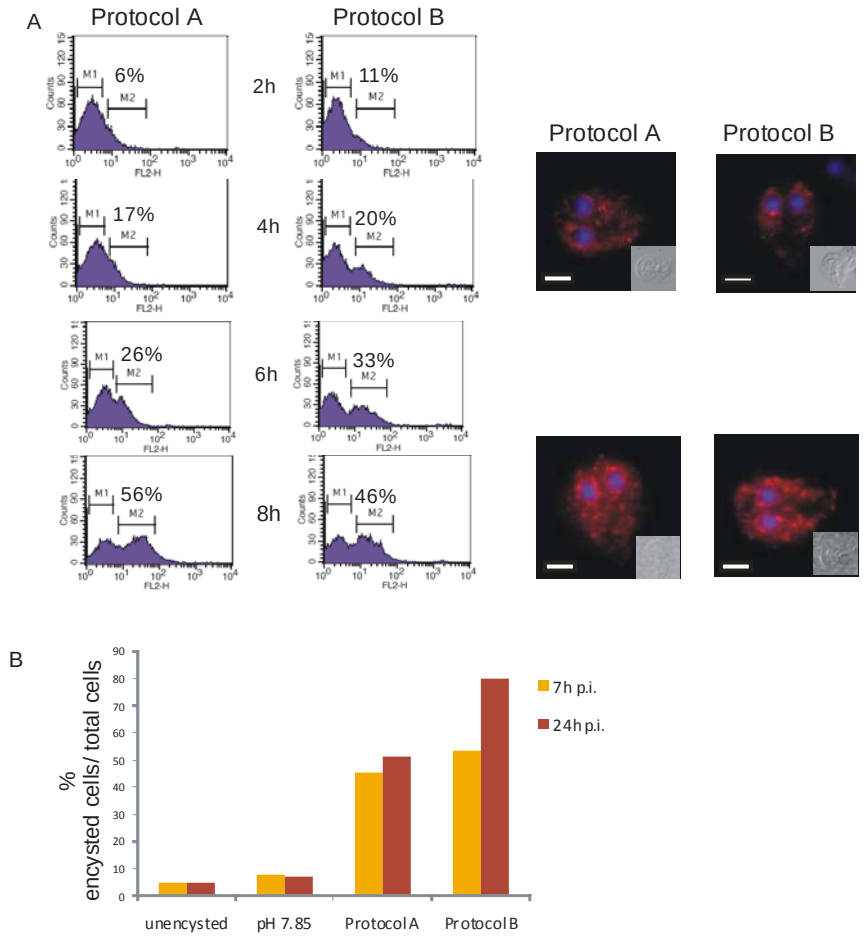


Figure 2 Comparison of the kinetics of the two encystation protocols. A) FACS analysis of encysting cells induced by protocol A (left column) or protocol B (right column). Chemically fixed and detergent permeabilized cells were labeled with an anti-CWP1 antibody coupled to Cy3. Gates for trophozoites (non-labeled, M1) and cells containing CWP1 in ESVs (M2) at four different time-points post induction are indicated together with calculated percentages of encysting cells. Representative IFA images of labeled cells (CWP1, red; DAPI, blue) are shown for the time points 4 h and 8 h p.i. Scale bars, 3µm. B) Quantitation of encystation at different conditions by microscopy. The percentage of encysting cells containing ESVs and cysts labeled with an anti-CWP1 antibody coupled to Cy3 at 7h (left column) and 24 h (right column) in each field of view was determined.

Comparative analysis of induced genes generated with two induction protocols reveals a small core set of bona fide encystation-specific genes

Microarray analysis of cells which were induced using protocol B at 7 h p.i. also yielded a relatively small set of 37 genes whose signal was increased >2 fold (Figure 3A). A rough categorization of the genes identified in the two 7 h datasets already indicates significant differences (Figure 3C). Most notable are the high proportion of hypothetical proteins and the identification of two different HCMps in the protocol B dataset.

Detailed comparative analysis of the upregulated genes in the two datasets revealed a greater number of divergent than intersecting positions (graphical depiction in Figure 3B). If we follow the definition that only genes which are significantly induced in both protocols are truly linked to encystation, the result suggests that each protocol produces major off-target effects. The intersecting dataset of 13 positions contained the all the confirmed, highly regulated encystation-specific genes including CWPs and Myb, however. The HCNCp identified in a previous study [17] was not included in any of the datasets, and the three upregulated HCMp family members are not encystation-specific according to these criteria. Genes in the intersecting dataset show a comparable degree of signal increase in both protocols with the exception of ORF 92729 (annotated as fatty acid elongase 1) which shows an induction difference of >2 fold between datasets. Induction of CWP3 appears to be independent of the protocol used. Conversely, induction of CWPs 1 and 2 is significantly lower with protocol A. This is consistent with the minor differences of CWP1 expression observed at early time-points in the comparative FACS analysis (Figure 2A), and could reflect differences in expression kinetics rather than the final amount of product.

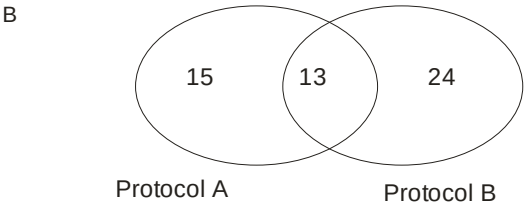
The results of this comparative analysis highlight the importance to distinguish between off-target effects and bona fide signals which induced differentiation. This is relevant as large datasets from genome-wide transcriptome analyses (SAGE and microarray) of differentiation processes (encystation/excystation) are available to the community via GiardiaDB, and information on stage-specific expression is included in the datasheet of each giardial gene. Biologically equally interesting, the direct comparative analysis of encystation with two distinct *in vitro* protocols allows to narrow down the region where encystation could be triggered in the host small intestine. Cultured parasites are exposed to complex changes of environmental conditions during encystation *in vitro*. Similarly, trophozoites which reside in the duodenal region experience strong temporal and local fluctuations of bile and pancreatic secretions, in addition to changes induced by dietary factors. Importantly, there is a considerable pH gradient between proximal and distal regions of the small intestine. Both *in vitro* protocols result in a high level of induction, suggesting that Giardia can filter out and transduce the signal for differentiation very effectively. The most consistent parameter of the two encystation protocols is the absolute requirement of a pH of 7.8 - 7.9. Because this appears necessary but not sufficient for strong induction of encystation *in vitro*, it seems unlikely that encystation *in vivo* is triggered in the duodenal region where the pH is 6 – 6.5. The lumen of the jejunum and the ileum, however, is increasingly alkaline and reaches pH 7 - 8 in distal regions. Cholesterol and fatty acids are resorbed by enterocytes in the small intestine, thus, the parasites in the distal regions of the ileum should encounter conditions which likely trigger differentiation.

Figure 3

A

		Protocol A	Protocol B
ORF number	Description	fold	fold
5435	cyst wall protein 2	24.7	15.1
5638	cyst wall protein 1	19.6	15.6
9046	Sugar transport family protein	9.1	
32657	Hypothetical Protein	8.5	11.9
8245	Glucosamine-6-phosphate deaminase	5.3	8.3
8722	Myb 1-like protein (gMyb2)	4.2	3.1
2421	Cyst wall protein 3	4.0	4.2
10552	Hypothetical Protein	3.7	3.5
14626	Oxidoreductase, short chain dehydrogenase/reductase family	3.3	5.8
137701	kinase, NEK	2.8	
92729	Fatty acid elongase 1	2.8	6.8
88581	Synapticglycoprotein SC2	2.7	2.4
12082	Hypothetical Protein	2.5	3.5
7134	Hypothetical Protein	2.4	
103785	Hypothetical Protein	2.4	
14759	6-phosphogluconate dehydrogenase	2.3	
16069	phosphoacetylglucosamine mutase	2.3	
7388	Hypothetical Protein	2.2	
112432	high cysteine membrane protein Group 5 (HCMp)	2.2	
88814	Protein kinase	2.2	
21924	kinase, NEK	2.2	
3063	Hypothetical Protein	2.2	3.9
9115	Glucose-6-phosphate isomerase	2.1	
4846	Protein 21.1	2.1	
106496	Hypothetical Protein	2.1	
7260	Aldose reductase	2.1	
24412	Protein 21.1	2.1	
102813	Protein 21.1	2.1	3.1
7139	Hypothetical Protein	2.0	
15250	high cysteine membrane protein Group 6 (HCMp)		8.8
9620	high cysteine membrane protein Group 2 (HCMp)		7.4
14259	Glucose 6-phosphate N-acetyltransferase		5.3
93488	Hypothetical Protein		4.3
5800	Hypothetical Protein		4.2
27028	Hypothetical Protein		3.8
8987	Hypothetical Protein		3.6
89849	Hypothetical Protein		3.5
5810	Hypothetical Protein		3.0
9355	Hypothetical Protein		3.0
35999	Hypothetical Protein		2.9
32419	Hypothetical Protein		2.8
37010	Hypothetical Protein		2.8
7982	UDP-glucose 4-epimerase		2.7

B



C

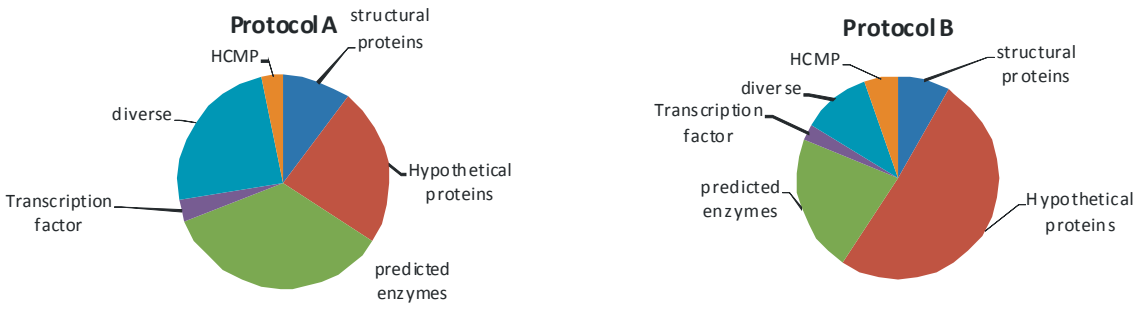


Figure 3 Comparative analysis of gene regulation induced by protocols A and B. A) The tabular depiction of ORFs with a statistically significant change of signal of >2.0 in the two datasets shows 13 common ORFs. B) Graphical representation (Venn diagram) of the two datasets. The intersecting positions are designated bona fide encystation-specific ORFs. C) Categorization of ORFs in the datasets shows that the protocol B data set contains significantly more hypothetical ORFs.

A myb binding sequence is a signature motif for encystation-specific genes

The *cis* acting elements for control of Giardia gene expression are normally included in short ~100 bp sequences upstream of transcription start sites. The only element common to many giardial promoters is a short stretch of AT-rich sequence (initiator) close to the transcription start site [27]. CWP promoters follow this rule, and in addition, also contain binding sites for the Myb2-like transcription factor. A sequence analysis of ~100 bp upstream of the 13 encystation-specific genes revealed that all promoters contained at least one MBS. Two promoters contained its reverse complement (ORFs 14626, 10552) and a strongly degenerate variant (T-T-A-C-A-A) in the case of ORF 10552. A MEME analysis using a motif-width between 4 and 12 nt showed that the MBS was the only motif which was over-represented in the promoter regions of encystation genes (Figure 4 A). Using this MEME analysis we identified the position and orientation of 33 MBS in 13 promoter sequences (~100 bp of upstream sequence) and generated a more refined MBS 6-mer motif representation (C/T-T/A-A-C-A/T-G/A) with this training set (Figure 4B). The result is fully consistent with that of the scanning mutagenesis experiment to analyze binding properties of Myb2 performed by Sun *et al*. [23]. We tested if this motif was also over-represented in the 39 (15 + 24) non-overlapping genes. This analysis revealed one MBS each in the promoter sequences of five additional genes that appeared either in the protocol A or B dataset at 7 h p.i., but were not included in the intersecting group because they were eliminated for technical reasons or because they fell just below the cut-off of 2.0 fold signal increase (Supporting material Figure S1). Nevertheless, after taking into account additional criteria such as statistical significance of upregulation of >0.9, appearance of the MBS in the MAST analysis, upregulation in the 3 h time-point, or mention in the literature [12,20], we decided to include these five candidates in a final set of 18 encystation-specific genes.

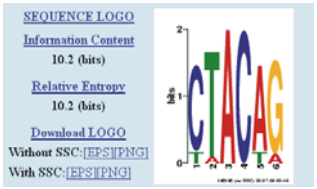
We expanded our analysis and found that none of the promoters of the 18 genes in the 45 min. time-point (protocol A) contained a MBS. However, in 16 of these promoters, at least one AT-rich motif was identified by a MEME/MAST analysis, predominately in the distal half of the promoter (Supporting material Figure S2 A). This is additional support for the idea that the early response to protocol A does not involve Myb. In the corresponding analysis of the 3 h time-point, a slightly divergent version of the MBS starts to emerge in a MEME motif search (Supporting material Figure S2 B). Six out of nine genes in this group are included in the final set of encystation-specific genes. Finally, a global MAST analysis of the promoter sequences of all 4969 genes in the Giardia genome found the 6 nt MBS sequence in 715 promoters (including the 18 bona fide encystation genes). This frequency of occurrence is bracketed by the predicted frequencies of the 6-mer (sense orientation) of 121 if no variability is allowed, and 1941 if we assume equal probability for two nucleotides at positions 1, 2, 5, and 6 in the motif. Together with the highly significant over-representation in the promoter regions of the encystation-specific genes, this strongly suggests an otherwise random frequency of occurrence of the MBS motif in upstream regions of genes.

Myb is constitutively expressed but upregulated in cells during encystation. Since its promoter also contains a MBS positive autoregulation [23] (Nicolaides 1991 MCB) is a likely mechanism to ensure rapid increases in Myb concentrations in differentiating cells. Sun et al. showed that three tandem MBS copies confer some upregulation to the constitutive Ran promoter in a reporter construct. Together with data demonstrating a *cis* acting sequence in the promoter of CWP2 conferring tight transcriptional repression of the gene in trophozoites [18], this fits with the idea that MBS modulates but does not control stage-specific transcription. Indeed, the promoters of two of our final set of 18 encystation genes contain no MBS on the sense strand. The need for rapid upregulation of Myb during encystation could also be explained by competition for space on the short promoter sequence.

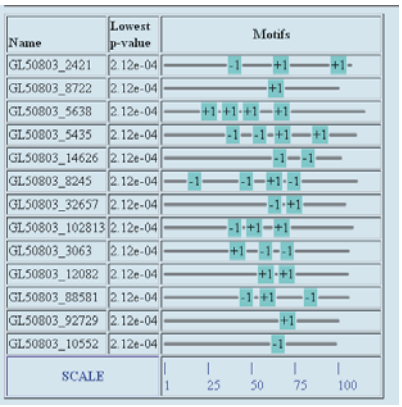
Taken together, the data strongly suggest that regulation of genes which are significantly induced during encystation at 7 h p.i. involves MBSs and giardial Myb2. The GS strain of *Giardia lamblia* has been reported to respond less efficiently to induction of encystation *in vitro*. As a possible explanation sequence differences in the promoter region of CWPs have been discussed [28]. To investigate this possibility further, we compared promoter sequences in the final dataset of encystation-specific genes. We found mutations in one or more MBS motifs in 9 of 14 promoters, which appear to confirm this trend (data not shown). The biological significance of this needs to be further examined, however.

Figure 4

A



B



C

GL50803_5638	Cyst wall protein 1
GL50803_5435	Cyst wall protein 2
GL50803_2421	Cyst wall protein 3
GL50803_8722	Myb 1-like protein
GL50803_92729	Fatty acid elongase 1
GL50803_88581	Synaptic glycoprotein SC2
GL50803_8245	Glucosamine-6-phosphate deaminase
GL50803_10552	Hypothetical Protein
GL50803_3063	Hypothetical Protein
GL50803_32657	Hypothetical Protein
GL50803_14626	Oxidoreductase
GL50803_12082	blasthit 1e-10 gi 46120805
GL50803_102813	Protein 21.1
GL50803_9046	Sugar transport family protein
GL50803_9115	Glucosamine-6-phosphate isomerase
GL50803_16069	phosphoacetylglucosamine mutase
GL50803_27082	Hypothetical Protein
GL50803_21924	Hypothetical Protein

Figure 4: Representation and distribution of the MBS in the promoter regions of regulated genes. A) MEME analysis and sequence logo of the MBS generated with the 13 encystation-specific promoters as a training set. A single change to the alternative nucleotide at positions 1, 2, 5, and 6 is tolerated. B) The MEME analysis shows also the position of MBS in the sense (+) and antisense (-) orientation in promoters. Position 1 on the scale indicates the most proximal nucleotide next to the start codon of the ORF. C) Tabular depiction of the final set of encystation-specific ORFs including five additional positions (dark blue).

Two hypothetical proteins are upregulated and localize to the ER

In addition to several known and predicted genes, four newly identified hypothetical encystation-specific genes are included in the final dataset. To determine if this group might contain structural proteins of the CW or the CW-plasma membrane interface we expressed the two predicted secreted proteins encoded by two ORFs (3063, 32657) as HA epitope-tagged variants under the control of their respective endogenous promoters (Figure 5). Consistent with the microarray data and with previous experiments using CWP sequences, a short ~100 bp promoter sequence was sufficient for a strongly stage-specific expression of both tagged proteins. The tagged products localized to the ER in transgenic encysting parasites (note the distinct nuclear envelope staining), and were found at the cell periphery, but not in the cyst wall, as well as in internal compartments of cysts by IFA (Figure 5A, B). This indicates that they are exported during establishment of the cyst wall, but the lack of distinct ESV staining suggests a separate transport route. ORF 32657 encodes a multipass transmembrane protein, the predicted ORF 3063 product is possibly a type 2 protein. Despite the completely different topologies, both have very similar localizations and expression kinetics. Taken together, our data demonstrates that no strongly expressed proteins in addition to the three CWP family members are integrated into the extracellular matrix that forms the cyst wall.

Figure 5

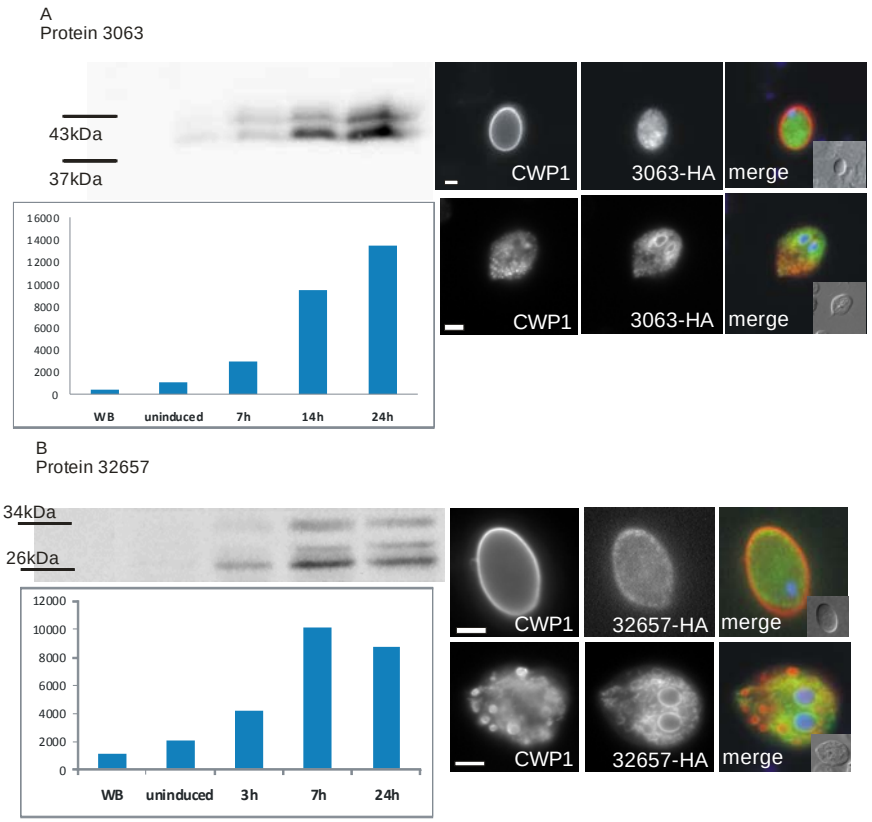


Figure 5 Expression of predicted secreted proteins as tagged variants in encysting cells. A, B) Expression of HA-tagged ORFs 3063 and 32657 is controlled by the respective endogenous promoters. Western analysis of protein extracts separated on SDS-PAGE shows strong induction of the proteins during encystation (0 – 24 h p.i.). IFA analysis of chemically fixed and detergent-permeabilized cells. Representative cells at 7 h (encysting trophozoites, top rows) and 24 h (cyst, bottom rows) are shown. CWP1, Red; 3063-HA, green; DAPI, blue. Scale bars, 3 µm.

MATERIAL AND METHODS

Biochemical reagents

Unless otherwise stated, all chemicals were purchased from Sigma and cell culture reagents were from GIBCO BRL.

Culture

Trophozoites of the *G. lamblia* strain WBC6 (ATCC catalog number 50803) were grown axenically in 11-ml culture tubes (Nunc, Roskilde, Denmark) containing Diamond's TYI-S-33 medium supplemented with 10% adult bovine serum and bovine bile at 37°C. Parasites were harvested by chilling the culture tubes on ice for 30 min to detach adherent cells and cells were collected by centrifugation at 1,000 x *g* for 10 min. Cells were then resuspended/washed in ice cold phosphate-buffered saline (PBS).

Induction of Encystation

Protocol A: Two-step encystation was induced as described previously (Boucher *et al* 1990, Gillin *et al* 1989). Briefly, cells were cultivated for 44h to confluency in bile free medium (preencysting medium) and subsequently in prewarmed encystation medium (preencysting medium with an increase in pH to pH 7.85 and addition of porcine bile (0.25 mg/ml, Sigma B8631) as well as lactic acid (0.545 mg/ml, Sigma L-200)) to induce encystation.

Protocol B: Lipid starvation was induced as described in (Lujan *et al* 1996). Briefly, preencysting medium containing delipidated FCS (PHM-L Lipsorb, Calbiochem) and an increased pH to pH 7.85 was used to induce differentiation of cells previously normally grown to confluency in complete medium.

Protein analysis

Protein analysis was performed as described in Stefanic *et al* 2009. Giardia parasites were harvested for gel electrophoresis by chilling culture tubes in ice and centrifugation at 1000 g. The cell pellet was washed once in ice-cold phosphatebuffered saline (PBS) and counted in a Neubauer chamber. The cell pellet was dissolved in SDS sample buffer to obtain $2*10^5$ cells in 50 µl and boiled for 3 minutes. Dithiothreitol (DTT) was added to 7.75 µg/ml before boiling. SDS-PAGE on 12% polyacrylamide gels and transfer to nitrocellulose membranes was done according to standard techniques. Nitrocellulose membranes were blocked in 5% dry milk, 0.05% TWEEN-20 in PBS and incubated with primary antibody, a biotinylated - anti-HA (high affinity, 3Flo, Roche), 1: 500 in blocking solution. Bound antibodies were detected with horseradish peroxidase conjugated streptavidin antibody 1: 1000 (Thermo Scientific Pierce Protein Research Products) and developed using Western Lightning Chemiluminescence Reagent

(PerkinElmer Life Sciences, Boston, MA). Data collection was done in a MultiImage Light Cabinet with AlphaEaseFC software (Alpha Innotech, San Leonardo, CA) using the appropriate settings.

Immunofluorescence analysis

Cells were harvested as described above. Fixation and preparation for fluorescence microscopy was done as described previously (Marti *et al*. 2003). Briefly, cells were washed with cold PBS and fixed with 3% formaldehyde in PBS for 40 minutes at room temperature (20°C), followed by a 5 minute incubation with 0.1 M glycine in PBS. Cells were permeabilized with 0.2% Triton X-100 in PBS for 20 minutes at room temperature and blocked overnight in 2% BSA in PBS. Incubation of all antibodies was done in 2% BSA, 0.2% Triton X-100 in PBS. Mouse monoclonal Alexa Fluor 488-conjugated anti-HA (Roche Diagnostics, Manheim, Germany; dilution 1:30) or Cy3-conjugated anti-CWP1 (Waterborne, New Orleans, LA; dilution 1:80) were incubated for 50min on ice. Washes between incubations were done with 0.5% BSA, 0.05% Triton X-100 in PBS. Labeled cells were embedded with Vectashield (Vector Laboratories, Burlingame, CA) containing the DNA intercalating agent 4_-6-diamidino-2-phenylindole (DAPI) for detection of nuclear DNA. Immunofluorescence analysis was performed on a Leica DM IRBE fluorescence microscope.

Fluorescence activated cell sorting

For quantification of cells expressing CWP1 and cyst yield, parasites were induced to encyst with either protocol A or protocol B for the required amount of time. Cysts and detached trophozoites were collected as described above, pelleted by centrifugation at 1000 *g* for 10 minutes and washed with PBS. Fixed and permeabilized cells were fluorescently labeled for 50 min on ice using monoclonal Cy3-conjugated anti-CWP1 antibody (Waterborne, New Orleans, LA; dilution 1:80). Before FACS analysis, cells were washed twice in PBS in resuspended in ice cold PBS. Unlabeled samples were used to determine background fluorescence, and subsequently, fluorescently labeled cysts were analyzed in triplicate on a FACS Calibur flow cytometer (Becton & Dickinson, Basel, Switzerland). All samples were analyzed in parallel by IFA to assess encystation efficiency and labeling and permeabilization quality.

Bioinformatic analysis

MEME/*MAST*: Motif Discovery and Search (*meme.sdsc.edu/)*

Microarray

Microarray was done as described in [29]. Briefly, for RNA extraction and microarray analysis cells were encysted if required and harvested as described above. RNA isolation was performed using the RNAeasy kit (Qiagen, Stanford, CA) following the "Animal Cells Spin" protocol. Residual genomic DNA was removed with DNAseI digestion according to the manufacturer's protocol. The integrity of the RNA was analyzed in a Bioanalyser (Agilent Technologies Inc., Palo Alto, CA) with "Eukaryote Total RNA Nano Series II" settings.

The slides used here were *Giardia lamblia* microarray version 2 (provided by JCVI free of charge). They are aminosilane surface coated glass slides with ss-oligo (70 mers) printed with a Genetix arraying machine. The array contains 19230 elements and covers the whole *Giardia lamblia* WB strain genome. For further information check *Giardia lamblia* Microarray Version2 (http://pfgrc.jcvi.org/index.php/microarray/array_description/giardia_lamblia/version2.html).

For dual channel microarray analysis, extracted total RNA was processed using the Amino Allyl MessageAmp™II a RNA Amplification Kit (Ambion, Austin, TX) and labelled with N-hydroxysuccinimidyl ester-derivatized reactive dyes Cy™3 or Cy™5, according to the manufacturer's protocol. After purification, 2 µg each of Cy3 or Cy5 labelled aRNA were denatured, added to SlideHyb™ Buffer I (Ambion), and hybridized to *G. lamblia* microarrays version 2 (TIGR) in a Tecan HybStation at the Functional Genomics Centre Zurich, Switzerland. The arrays are aminosilane surface coated glass slides with 9115 oligonucleotides (70mers) designed to cover the whole *G. lamblia* WBC6 strain genome.

Prior to hybridization, slides were hydrated and blocked with 150 µl BSA-Buffer (0.1 mg/ml BSA, 0.1 % SDS in 3 x SSC-Buffer) for 1h at 50°C. After washing, samples were injected and hybridized for 16 h at 42°C. Slides were scanned in an Agilent Scanner G2565AA, using laser lines 543 nm and 633 nm for excitation of Cy3 and Cy5, respectively. Spatial scanning resolution was 10 µm, single pass. The scanner output files were quantified using the Genespotter Software (MicroDiscovery GmbH, Berlin, Germany) with default settings and 2.5 µm radius. The median spot intensities were evaluated with the Web application MAGMA [30] and normalized using the print-tip-wise loess correction of the *limma* package (Smith, 2005). Potential gene-specific dye-effects were estimated from self-self hybridizations. Differential expression of genes is reported as the fold-change compared with control treated samples, as well as the p-value for differential expression as estimated by the empirical Bayes model implemented in *limma*. All reactions were performed in triplicate.

Expression vector construction and transfection

Stable chromosomal integration of the described constructs was performed using the pPacV-Integ expression vector [31] using *XbaI* and *Pac*I restriction

sites. Oligonucleotides (5'-3' orientation) used in this study were:
3063p_XbaI_s CGTCTAGATGGGTCGTCGGCTCTACAG,
3063p_HA_EcoRI_as,
CGGAATTCCGCGTAGTCTGGGACATCGTATGGGTATCCGTCTCCCGTAGACAG,
3063_EcoRI_s CGGAATTCAAGCCTCCAGTAGTGCCGTC,
3063_PacI_as
CGTTAATTAATTACAGATTTTTACTAGTAGGGCAG,
32657_s
GCTAGCGGATTGTGACTCTTGTATTAGGTG,
32657_HA_as
TTAATTAACTACGCGTAGTCTGGGACATCGTATGGGTAATCCATTGTTGCGTACGAG. If required plasmid vector DNA was linearized using SwaI restriction enzyme and approx. 15 µg of digested plasmid DNA was electroporated (350V, 960µF, 800Ω) into trophozoites and integration occurs by homologous recombination under selective pressure with the antibiotic puromycin

Acknowledgements

The *Giardia lamblia* microarrays (version 2) were kindly offered through NIAID's Pathogen Functional Genomics Resource Center, managed and funded by Division of Microbiology and Infectious Diseases, NIAID, NIH, DHHS and operated by the J. Craig Venter Institute. The Functional Genomics Centre Zürich, Switzerland (www.fgcz.uzh.ch) is a joint facility of the ETHZH and the University of Zürich and in particular Dr. Weihong Qi from the FGCZ for her contribution. We are grateful to the "Stiftung zur Förderung der Wissenschaftlichen Forschung an der Universität Zürich" for financial support for this project. C.S. was supported by the Roche Research Foundation, Research in the Hehl lab is supported by SNF grant #3100A0-112327.

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SUPPLEMENTARY MATERIAL

Figure S1 List of the five additionally defined *bona fide* encystation-specific genes and the criteria they fulfill.

S1

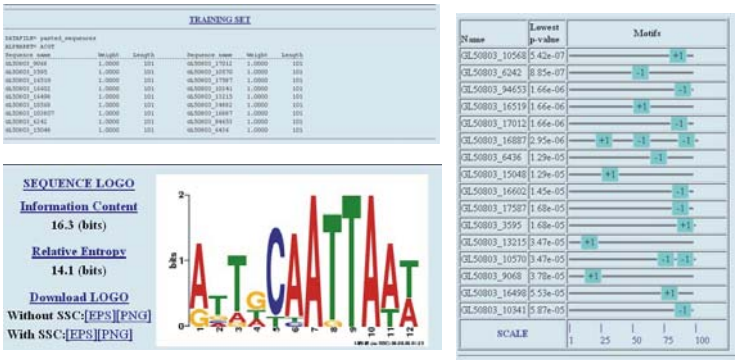
ORF	Gene Description	fold: Protocol A	fold: Protocol B		present in
GL50803_9046	Sugar transport family protein	9.14	technical issue	3h up	MAST
GL50803_9115	Glucosamine-6-phosphate isomerase	2.14	technical issue	Sun *et al.* 2002	MAST
GL50803_16069	phosphoacetylglucosamine mutase	2.26	technical issue	Lopez *et al*. 2003	MAST
GL50803_27082	Hypothetical Protein	1.99	3.79		MAST
GL50803_21924	Hypothetical Protein	2.16	1.92		MAST

Figure S2 MEME and MAST analyses of promoters upregulated at early time points. An A/T-rich sequence is the most frequently identified motif in the 45 min. dataset. In the 3 h dataset the MBS motif emerges in 9 of 16 ORFs.

S2

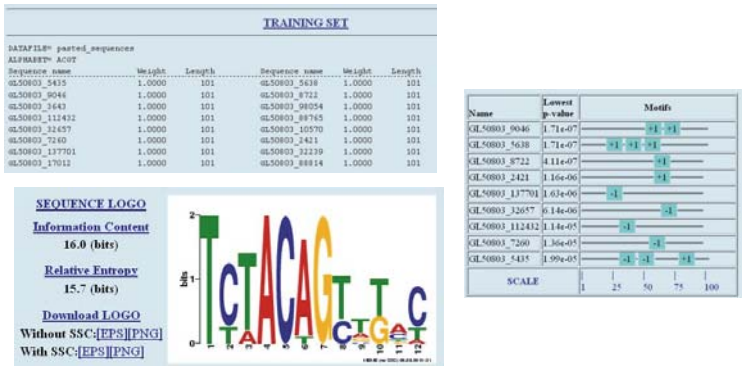
A

45min
Protocol A



B

3h
Protocol A



PART IV

Manuscripts

3) Epigenetic mechanisms regulate stage differentiation in the early divergent protozoan *Giardia lamblia*

Sabrina Sonda*, **Laura Morf***, Iveta Bottova, Hansruedi Baetschmann, Hubert Rehrauer, Mohamed-Ali Hakimi and Adrian B. Hehl

**These authors contributed equally to this work,* Molecular Microbiology, accepted January 2010

This study represents a major part of my work of the third year of my PhD. I was substantially involved in all parts of this study including the establishment of experimental procedures, development of ideas as well as the result interpretation. Apart from the bioinformatical analyses, I did the majority of experimental work (see Figure 1, 3a, 4a and b, 5, 7, 8a, b and d, 9). Further, I was closely involved in defining the exact content and structure of the manuscript.

The combined results of the study (2) "Differentiation in encystation in *Giardia lamblia* is determined by small set of stage-regulated genes" and this study resulted in a follow up project performed by a PhD student (Petra Wampfler) in our laboratory.

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Epigenetic mechanisms regulate stage differentiation in the minimized protozoan *Giardia lamblia*

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Summary

Histone modification is an important mechanism regulating both gene expression and the establishment and maintenance of cellular phenotypes during development. Regulation of histone acetylation via histone acetylases and deacetylases (HDACs) appears to be particularly crucial in determining gene expression patterns. In this study we explored the effect of HDAC inhibition on the life cycle of the human pathogen *Giardia lamblia*, a highly reduced parasitic protozoan characterized by minimized cellular processes. We found that the HDAC inhibitor FR235222 increased the level of histone acetylation and induced transcriptional regulation of ~2% of genes in proliferating and encysting parasites. In addition, our analyses showed that the levels of histone acetylation decreased during differentiation into cysts, the infective stage of the parasite. Importantly, FR235222 treatment during encystation reversed this histone hypo-acetylation and potently blocked the formation of cysts. These results provide the first direct evidence for epigenetic regulation of gene expression in this simple eukaryote. This suggests that regulation of histone acetylation is involved in the control of *Giardia* stage differentiation, and identifies epigenetic mechanisms as a promising target to prevent *Giardia* transmission.

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Introduction

Regulation of gene expression is a complex process controlled by several molecular mechanisms including modification of chromatin structure, activity of sequence-specific DNA binding proteins, and post-transcriptional modulation of mRNA levels. Epigenetic histone modifications result in alteration of chromatin structure, which in turn induces changes in gene expression (reviewed in Jenuwein and Allis, 2001; Kouzarides, 2007). In general terms, the process of covalent histone acetylation regulates gene expression by modifying DNA packaging, with histone hyperacetylation leading to chromatin decondensation and activation of transcription. Conversely, histone hypoacetylation leads to a tighter DNA-histone binding, with consequent chromatin condensation and gene silencing. The level of histone acetylation is strictly controlled by the concerted activity of histone acetylases (HATs) and deacetylases (HDACs), which act as central organizers of chromatin remodelling and gene transcription. The correct regulation of HDACs is critical for cellular homeostasis. The importance of a tight control of HDAC activity is exemplified by its upregulation associated with uncontrolled cell growth at the onset of cancer (Glozak and Seto, 2007). In this context, considerable efforts have been invested to develop inhibitors of HDAC activity (HDACi) as anti-cancer drugs, as HDACi were shown to counteract aberrant cell cycle regulation in cancer cells both *in vitro* and *in vivo* (Bolden *et al.*, 2006). In addition, recent studies revealed that the activity of HDACs is crucial not only for cell cycle regulation but also for the orchestration of development and cell differentiation in different mammalian organs (Margueron *et al.*, 2005; Haumaitre *et al.*, 2009). Despite these recent advances, our understanding of the roles HDACs play remains limited.

In this study we investigated whether HDAC activity also plays a role in the differentiation process of the intestinal pathogenic parasite *Giardia lamblia*, a protozoan characterized by a highly reduced genome and significant minimization of most cellular systems due to secondary

reduction (Morrison *et al.*, 2007). In this respect, the parasite is a useful model organism to study basic biological processes of higher eukaryotes. The *G. lamblia* life cycle is comprised of highly motile flagellated trophozoites and quiescent cyst surrounded by a protective cyst wall (Gerwig *et al.*, 2002). Differentiation from the trophozoite to the cyst stage is critical for the parasite survival in the environment and for disease transmission.

Giardia lamblia encystation is a complex process that involves both expression of specific proteins and also changes in the parasite's metabolism (Adam, 2001). The encystation process begins with an early phase where cyst wall components, including cyst wall proteins (CWPs) 1–3, are synthesized and accumulated in encystation-specific secretory vesicles (ESVs). ESVs are formed transiently only during the time of encystation and display Golgi-like characteristics (Lujan *et al.*, 1998; Marti and Hehl, 2003; Hehl and Marti, 2004; Stefanic *et al.*, 2009), allowing both maturation and export of the CWPs to the cell exterior to form the cyst wall. This late phase is completed with the sequential secretion of two fractions of cyst wall material to the parasite surface 20–24 h after induction of encystation, where it polymerizes to a rigid extracellular matrix, the cyst wall.

Despite recent discoveries in the field of *G. lamblia* encystation, our understanding of the molecular mechanisms initiating and regulating gene expression during this critical process remains incomplete. Several factors have been implicated, including kinase and phosphatase-mediated signal transduction (Ellis *et al.*, 2003; Bazan-tejeda *et al.*, 2007; Lauwaet *et al.*, 2007; Pan *et al.*, 2009), arginine deiminase activity (Touz *et al.*, 2008), proteinases (Touz *et al.*, 2002a) and dipeptidyl peptidase IV (Touz *et al.*, 2002b). In addition, we recently showed that the synthesis of the sphingolipid glucosylceramide is also essential for complete cyst formation in *G. lamblia* (Stefanic *et al.*, 2009). However, much less is known about transcriptional regulation of encystation-specific genes. To date only a few putative encystation-related transcription factors have been identified and partially characterized in *G. lamblia*: a Myb2 homologue (Sun *et al.*, 2002; Huang *et al.*, 2008), two members of the GARP family (Sun *et al.*, 2006), one ARID family member (Wang *et al.*, 2007) and a recently described WRKY protein (Pan *et al.*, 2009). All these transcription factors, whose expression increases during encystation, bind to the short promoter of encystation-induced genes and act as *trans*-activators of transcription. In addition to *trans*-activating factors, mechanisms for negative regulation have also been suggested to control CWP expression in vegetative trophozoites, including regulation of mRNA stability via the 3′ untranslated region (UTR) (Hehl *et al.*, 2000), the nonsense-mediated mRNA decay (NMD) factor UPF1 (Chen *et al.*, 2008), and presence of negative *cis*-acting elements in the CWP promoters (Davis-Hayman *et al.*, 2003).

Because *G. lamblia* encystation is associated with clearly detectable changes in mRNA levels and the degree of histone acetylation is critical for transcriptional control, we hypothesized that epigenetic chromatin modifications via histone acetylation may participate in the modulation of stage differentiation in this parasite. To test this, we analysed whether modifying histone acetylation levels by inhibiting giardial HDAC affected parasite encystation.

Results

Histone acetylation decreases during G. lamblia *stage conversion*

Giardia lamblia encystation is regulated at the transcriptional level and results in high levels of transcription of encystation-specific genes during the first 7–9 h post induction. To investigate whether epigenetic mechanisms dependent on histone acetylation are involved in the stage conversion of *G. lamblia*, we compared the histone acetylation levels in trophozoites and encysting cells. To test this, we incubated isolated parasites with an anti-acetyl lysine antibody after chemical fixation and detergent permeabilization. Immunofluorescence analysis revealed that the labelling was restricted to the nuclei and that it overlapped with DAPI-stained DNA, as expected for labelled histones (Fig. 1A). As previously shown for the labelling of methyl-modified histones (Dawson *et al.*, 2007), the two giardial nuclei were similarly stained, indicating that the chromatin of both nuclei can be modified by the acetylation mark. Importantly, no cross-reaction was observed with cytosolic acetylated proteins, including tubulin (Fig. 4B), indicating that the antibody exclusively recognizes acetylated proteins restricted to the nucleus.

Next we quantified the degree of histone acetylation in trophozoites and encysting cells by flow cytometry. Our analysis showed that the staining intensity decreased in differentiating trophozoites (Fig. 1B), suggesting that the levels of histone acetylation are regulated in the parasite in a stage-specific manner.

Characterization of G. lamblia *HDAC*

As the cellular levels of histone acetylation are maintained by the concerted activity of HAT and HDAC enzymes, we investigated whether these enzymes are present in the parasite.

Analysis of the *Giardia* Genome Database (http://giardiadb.org) revealed the presence of putative enzymes responsible for histone modification, including HDACs (Table 1). In mammalian cells, the classical HDAC family consists of the ubiquitously expressed class I (HDACs 1–3, 8) and the tissue-specific classes II (HDACs 4–7, 9,

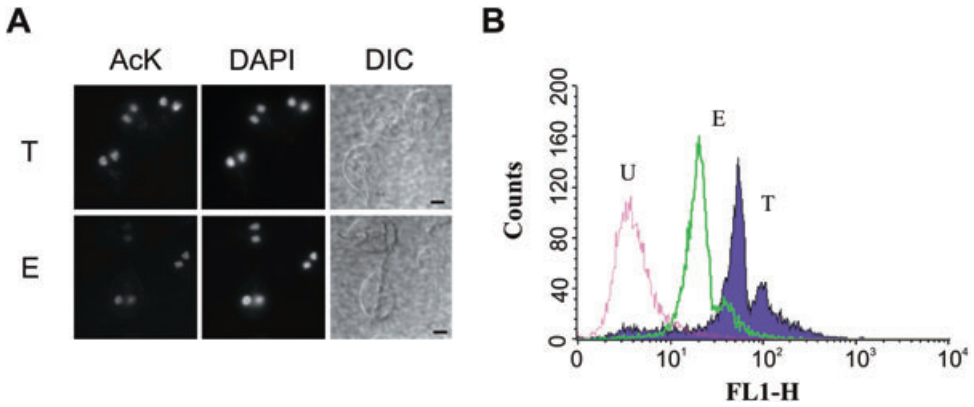

Fig. 1. Acetylation levels decrease in *G. lamblia* upon induction of encystation. Trophozoites (T) and parasites induced to encyst for 16 h (E) were probed for acetylated lysine (AcK).
A. Immunofluorescence analysis showing the staining restricted to the nuclei in both stages of the parasite. DAPI, nuclear staining; DIC, differential interference contrast. Scale bar: 3 μm.
B. Overlay histogram of the FACS analysis showing trophozoites (T) and encysting cells (E) stained for AcK. U, unstained cells. Note the decreased intensity of AcK staining in encysting cells compared with trophozoites (logarithmic scale on *x*-axis).

10) and IV (HDAC11), while the structurally unrelated class III is composed of sirtuin proteins 1–7. Interestingly, the *G. lamblia* genome codes for only one predicted homologue of the classical HDAC family, and four additional predicted sirtuin type 2 family homologues.

Multiple sequence alignments showed a high level of similarity between the single giardial HDAC and orthologues of protozoan and metazoan species (Fig. 2 and Table 2), indicating that the protein is highly conserved in this parasite. Importantly, the amino acids thought to be critical for human HDAC1 activity, including the residues in the catalytic pocket that co-ordinate binding to the co-factor Zn^{2+} and that are in contact with the HDACi Trichostatin A (TSA) (Finnin *et al.*, 1999; Vannini *et al.*, 2004), are also present in the giardial HDAC. Interestingly, the predicted giardial HDAC does not possess an AT insertion which is typical of Apicomplexa (Bougdour *et al.*, 2009), but contains a unique four-residue insertion in position 283–286, which is not found in other species.

Table 1. Putative histone modifying enzymes in *G. lamblia*.

Histone acetylases	
GL50803_10666	Histone acetyltransferase GCN5
GL50803_2851	Histone acetyltransferase MYST2
GL50803_16639	Histone acetyltransferase Elp3
GL50803_14753	Histone acetyltransferase type B subunit 2
GL50803_17263	Histone acetyltransferase MYST1
Histone deacetylases	
GL50803_3281	Histone deacetylase I
GL50803_10707	NAD-dependent histone deacetylase Sir2
GL50803_10708	Hypothetical protein, Sir2 domain
GL50803_16569	Transcriptional regulator, Sir2 family
GL50803_6942	Sir2 family protein
GL50803_11676	Sirtuin type 2
Histone methylases	
GL50803_8921	Set-2, putative
GL50803_13838	Hypothetical protein
GL50803_13790	Hypothetical protein
GL50803_9130	Histone methyltransferase HMT1
GL50803_17036	Hypothetical protein
GL50803_221691	Histone methyltransferase HMT2

To analyse the localization of giardial HDAC, a recombinant variant fused to a C-terminal HA tag was expressed in the parasites. Immunofluorescence analysis of transgenic cells revealed that the protein localizes to the parasite nuclei, suggesting that the giardial HDAC is likely to function in this subcellular location (Fig. 3A).

To gain further insights into the structure-function basis of giardial HDAC, a homology model was generated using the recently resolved structure of human HDAC8 as a template (Fig. 3B) (Vannini *et al.*, 2007). The predicted 3D structure revealed a striking similarity with the human homologue. In addition, position and orientation of the active site residues are essentially identical in the giardial and human structures, further supporting the predicted catalytic activity of *G. lamblia* HDAC.

To investigate whether the catalytic pocket of giardial HDAC could accommodate HDACi, we modelled the structure of the protein in complex with the cyclic tetrapeptide FR235222 (Mori *et al.*, 2003), a potent inhibitor that was recently shown to specifically target HDAC3 in the Apicomplexan parasite *Toxoplasma gondii* (Bougdour *et al.*, 2009) (Fig. 3C). The 3D structure of FR235222 was built using the values of the backbone dihedral angles as they are in the NMR solution conformation (Rodriquez *et al.*, 2006). Three binding modes of FR235222 in the homology model of *G. lamblia* HDAC were generated by docking using the program WITNOTP (Armin Widmer, Novartis Pharma, Basel, Switzerland). In all binding modes, the long side-chain of FR235222 was positioned in the deep pocket of HDAC with the ketone moiety at the tip of the side-chain

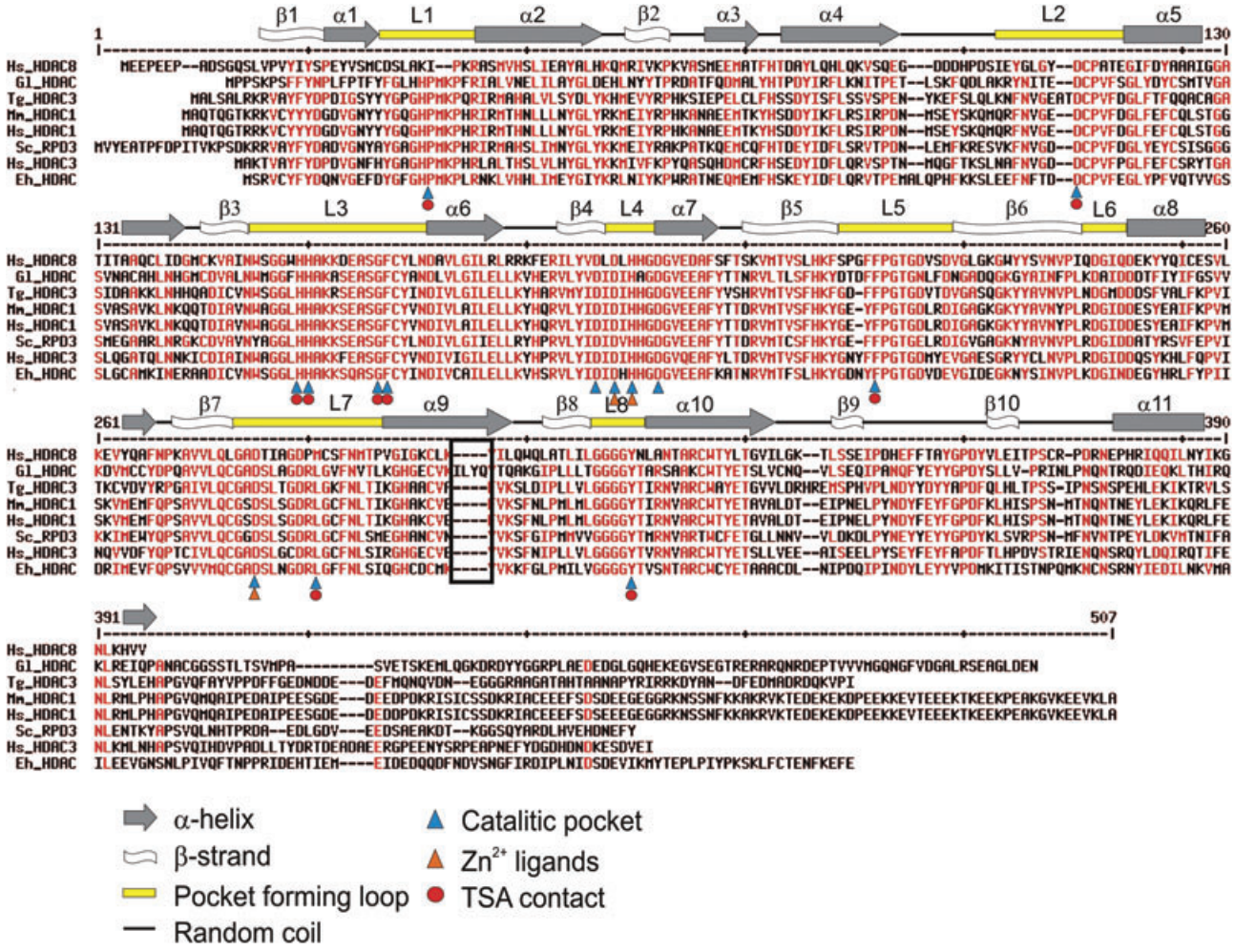

Fig. 2. Multiple sequence alignment of selected deacetylases. Protein sequence alignment of giardial HDAC with selected orthologues. Indicated are the residues important for catalytic activity and involved in zinc and TSA binding, based on crystal structure of related HDAC (Finnin *et al.*, 1999; Vannini *et al.*, 2004). The four residues of the *G. lamblia*-specific insertion are boxed. Alignments were performed with Multalin (http://bioinfo.genotoul.fr/multalin/). Gene bank accession numbers: GlHDAC (AAU89077), TgHDAC3 (AAY53803; ToxoDB accession no. 42.m00014), EhHDAC (AAV33348), HsHDAC1 (CAG46518), HsHDAC3 (NP_003874.2), HsHDAC8 (AF245664), MsHDAC1 (AAI08372), ScRPD3 (AAB20328).

Table 2. Sequence comparison of GI HDAC with selected orthologues.

Name	Identity (%)	Similarity (%)	AA overlap
Tg_HDAC3	48	68	376
Eh_HDAC	46	64	377
Mm_HDAC1	50	72	368
Hs_HDAC1	50	72	368
Sc_RPD3	51	69	372
Hs_HDAC3	45	66	432
Hs_HDAC8	41	62	368

G. lamblia, GlHDAC (AAU89077); *T. gondii* TgHDAC3 (AAY53803); *E. hystolitica*, EhHDAC (AAV33348); *H. sapiens*, HsHDAC1 (CAG46518); HsHDAC3 (NP_003874.2); HsHDAC8 (AF245664); *M. musculus*, MsHDAC1 (AAI08372); *S. cerevisiae*, ScRPD3 (AAB20328).

chelating the zinc. The three binding modes differ from each other in the orientations of the phenylalanine, isovaline, and 4-methyl-proline side-chains on the rim of the deep pocket. Each binding mode was energy minimized with rigid protein and zinc atom using the TAFF force field (Clark *et al.*, 1989). Upon energy minimization there is good surface complementarity in all of the three binding modes. Furthermore, the binding energy is similar in the three minimized structures, which is consistent with the heterogeneity of orientations observed for a set of inhibitors of human HDAC8 (Dowling *et al.*, 2008).

Inhibition of HDAC increases histone acetylation levels in G. lamblia

Because the stage conversion of *G. lamblia* correlated with decreased levels of histone acetylation, we investi-

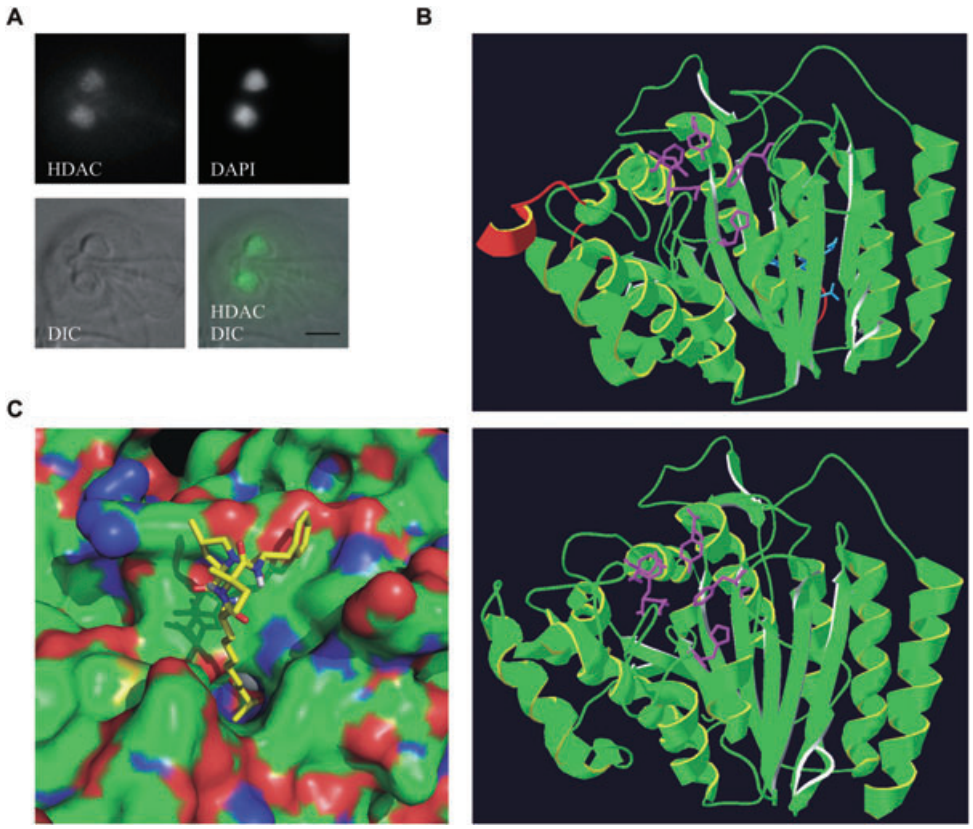

Fig. 3. Characterization of *G. lamblia* HDAC.
A. Fluorescence analysis of parasites expressing recombinant giardial HDAC fused to a C-terminal HA tag and encysted for 16 h. Cells were co-stained for nuclei (DAPI). DIC, differential interference contrast image. Scale bars: 3 μm.
B. 3D homology model of GlHDAC (upper panel) built with the SWISS-MODEL program and X-ray structure of HsHDAC8 (lower panel) used as a template. Key residues of the active-site are violet (Asp 101, His 142, Phe 152, Phe 208, Tyr 306 in HsHDAC8; Asp 94, His 135, Phe 145, Phe 201, Tyr 303 in GlHDAC). The four amino acids of GlHDAC insertion are blue. The GlHDAC region not covered by the HsHDAC8 template is red.
C. Predicted binding mode of FR235222 (sticks) in the homology model of GlHDAC (surface rendering). The energy minimization was performed with rigid protein. The atoms of FR235222 are coloured according to atomic element with carbon in yellow, nitrogen in blue, oxygen in red, and hydrogen in grey. Hydrogen atoms on the side-chains are not shown to avoid overcrowding. The polar surface of HDAC is coloured according to atomic element (nitrogen in blue and oxygen in red) while the non-polar surface is green. The zinc atom is the white sphere that is visible only partially at the bottom of the deep pocket close to the centre of the image. The long side-chain of FR235222 fits nicely in the cavity, and the two oxygen atoms at its tip chelate the zinc atom. Figure prepared with the program PyMOL (DeLano Scientific, USA).

gated whether reversing the decrease in acetylation by inhibiting HDAC could prevent parasite encystation. To this aim, we tested in the parasite the inhibitory activity of FR235222, the potent HDACi which was predicted to fit in the active site of *G. lamblia* HDAC in our 3D model. Immunofluorescence and quantitative analysis by flow cytometry revealed that treatment with FR235222 increased acetylation levels in encysting parasites (Fig. 4A). Selected subtypes of HDACs have been shown to target cytosolic non-histone proteins, including tubulin (Schemies *et al*., 2009). In our experiments, FR235222 treatment did not increase the acetylation levels of tubulin (Fig. 4B), implying not only that the inhibitor is not affecting the activity of other cellular deacetylases in a non-specific manner, but also that the single giardial HDAC is likely not able to accept tubulin as a substrate. In addition, nuclear basic proteins were isolated by acid extraction and probed for acetylation (Fig. 4C). Distinct acetylated proteins were identifiable in the region of 11–16 kDa, corresponding with the predicted masses of histone proteins in *Giardia* (Triana *et al*., 2001). Importantly, their acetylation level specifically increased in the presence of FR235222. Histone identities of the bands in the 11–16 kDa region were further confirmed by mass spectrometry analysis (Fig. S1).

Collectively, our data provide strong evidence that treatment with the HDACi FR235222 induced histone hyperacetylation in the parasite.

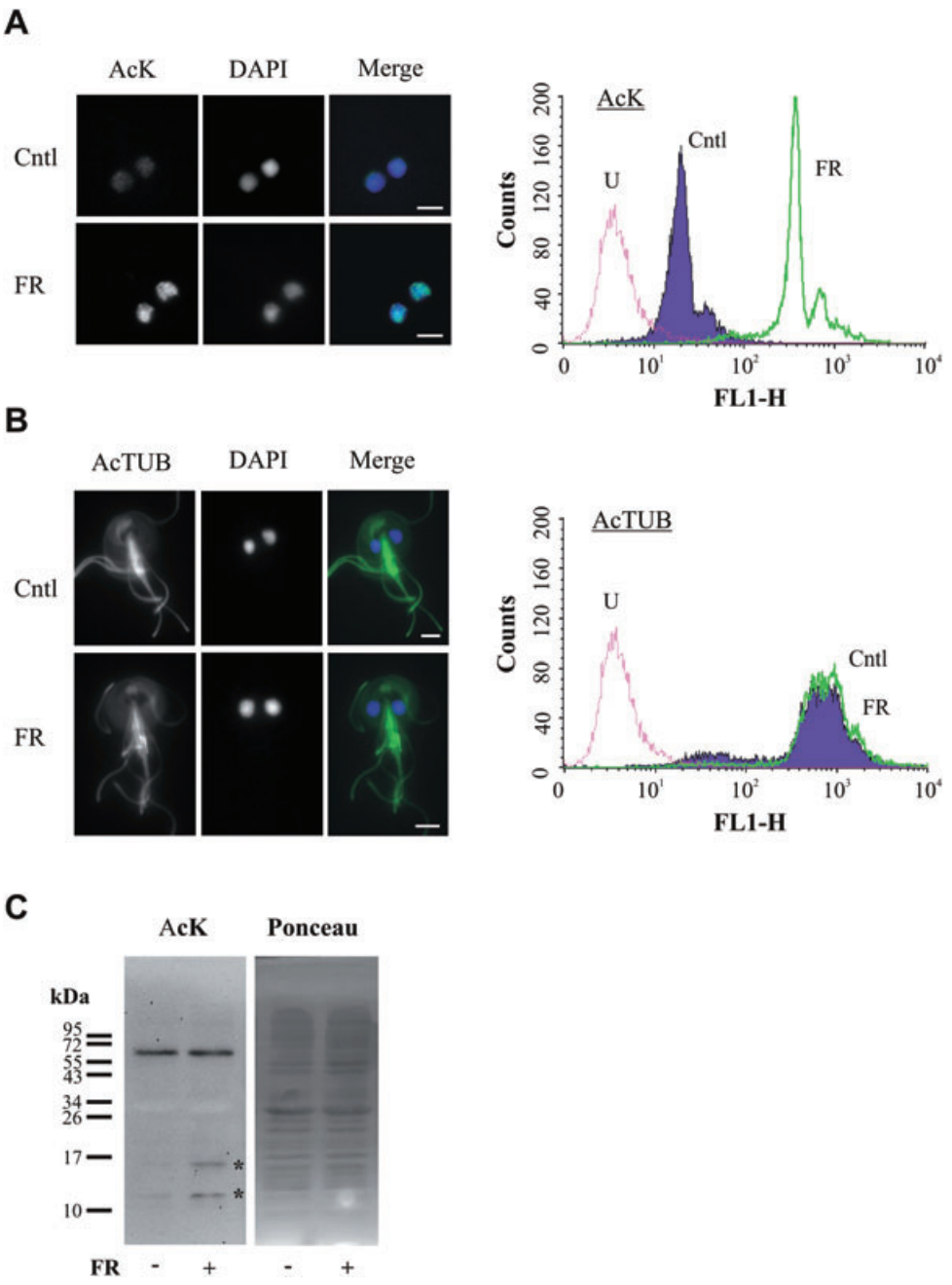

Fig. 4. FR235222 treatment increases acetylation levels of encysting cells.
A and B. Encysting parasites were treated with 2 μM FR235222 (FR) or solvent (Cntl), as described. Cells were analysed by immunofluorescence (left panels) after staining with anti-acetylated lysine (AcK) (A) or anti-acetylated tubulin (AcTUB) antibodies (B). DAPI, nuclear staining. Scale bar: 3 μm. The level of AcK and AcTUB upon FR235222 treatment was quantified by flow cytometry (right panels). Overlay histograms showing cells unstained (U), treated with solvent (Cntl) or 2 μM FR235222 (FR). Note the increased AcK signal in presence of the drug.
C. Encysting cells were treated with 2 μM FR235222 (FR) or solvent. Aliquots of 16 μg of acid-extracted proteins were separated by SDS-PAGE and probed with anti-acetylated lysine antibody. Note the increased acetylation of proteins with the predicted mass of giardial histones (asterisks) in presence of the HDAC inhibitor.

FR235222 inhibits expression of cyst wall proteins in encysting G. lamblia

Having shown that FR235222 treatment induces histone hyper-acetylation in *G. lamblia*, we next evaluated whether increased acetylation levels prevented parasite stage conversion to cysts. Induction of encystation results in high levels of expression of the main structural CWPs. Immunofluorescence analysis of CWP1 expression in induced cultures revealed a considerably decreased

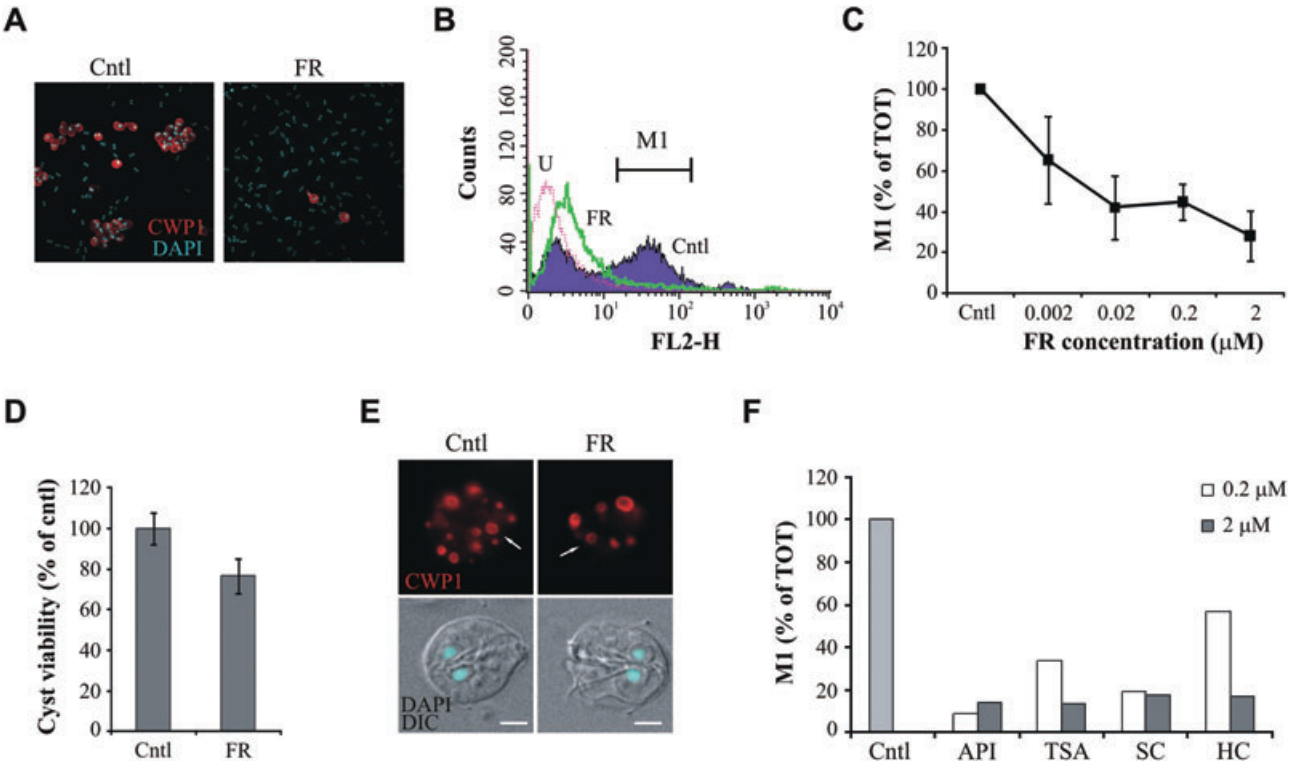

Fig. 5. FR235222 treatment inhibits *G. lamblia* encystation.
A. Encysting parasites were treated for 24 h with 2 μM FR235222, as described in the *Experimental procedures* section. Cells were stained with anti-cyst wall protein 1 (CWP1) antibody without permeabilization and analysed by immunofluorescence. Note the decreased number of cysts in the treated sample. DAPI, nuclear staining.
B. Encysting parasites were treated as in (A), permeabilized and stained for CWP1 followed by flow cytometry quantification. Overlay histogram showing cells unstained (U), treated with solvent (Cntl) or 2 μM FR235222 (FR).
C. Encysting parasites were treated with the indicated concentrations of FR235222 (FR) or solvent (Cntl), stained for CWP1 after permeabilization and analysed by flow cytometry. The amount of cells with M1 fluorescence is expressed as percentage of total cell number (TOT). Note the dose-dependent decrease in CWP1 expression upon drug treatment.
D. Viability of cysts following drug treatment was tested by trypan blue exclusion. Results of a representative experiment are presented as percentage of control ± standard errors ($n = 3$).
E. Immunofluorescence analysis of treated parasites as in (B). Note the normal morphology of encystation-specific vesicles (ESV, arrow) containing CWP1 in both treated and control samples. DAPI, nuclear staining; DIC, differential interference contrast. Scale bar: 3 μm.
F. Encysting parasites were treated with the indicated concentrations of HDAC inhibitors apicidin (API), Trichostatin A (TSA), scripaid (SC) and HC-toxin (HC) and CWP1 expression was quantified by flow cytometry after permeabilization. The amount of cells with M1 fluorescence is expressed as percentage of total cell number (TOT). The analysis revealed that all the compounds tested inhibited the expression of CWP1.

number of cysts in the inhibitor-treated sample compared with control cells (Fig. 5A). In addition, the few cysts produced in presence of FR235222 were less viable than the control sample (Fig. 5D). To determine whether the low number of cysts was due to a reduced CWP1 expression or a defect of protein secretion and consequent accumulation in the cell interior, we quantified the protein expression in detergent-permeabilized cells. Population-wide analysis by flow cytometry showed that FR235222 severely impaired expression of CWP1 (Fig. 5B). Interestingly, while the maximal inhibition of CWP1 expression was obtained by pre-treating the cells with FR235222 prior to induction of encystation, a substantial decrease of CWP1 protein level was also observed in the absence of drug pre-treatment, indicating a rapid effect of the inhibitor on CWP1 synthesis (Fig. S2).

Next, we performed a dose–response analysis to determine the potency of the FR235222-mediated inhibition of *G. lamblia* encystation. Flow cytometry quantification revealed that FR235222 was effective in inhibiting CWP1 expression already at low nanomolar concentration (Fig. 5C).

In addition, we tested whether FR235222 treatment not only inhibited CWP1 expression, but also altered the protein's intracellular distribution, a phenotype we recently described when parasite encystation was blocked following inhibition of sphingolipid synthesis (Štefanić *et al.*, submitted). Immunofluorescence analysis of encysting parasites showed that both in control cells and in the minor proportion of FR235222-treated cells with detectable CWP1 levels, the protein was mainly localized in ESVs with typical morphology, indi-

cating that formation of these compartments was not compromised (Fig. 5E).

To further confirm that the FR235222 inhibition of *G. lambia* encystation was indeed a specific effect, additional known HDACi, i.e. apicidin, TSA, scripaid and HC-toxin, were tested. All the inhibitors were highly effective in reducing CWP1 expression, as assessed by flow cytometry analysis (Fig. 5F) and enumeration of CWP1-expressing parasites by manual counting (Fig. S4). Collectively, our data indicate that exposure of encysting cells to HDACi severely compromised the encystation process by preventing induction of CWP expression.

FR235222 treatment does not downregulate the expression of constitutively expressed proteins

Next we asked whether FR235222-mediated downregulation of protein expression took place at a global level or it was specific for encystation-induced protein. We tested this by quantifying the degree of regulation of constitutively expressed proteins. The cellular levels of three unrelated endogenous proteins [namely the small GTPase Sar1, the endoplasmic reticulum-resident protein disulphide isomerase 2 (PDI2) and clathrin], which are involved in the secretory transport of CWP protein but whose expression is not stage-regulated, were monitored in encysting cells treated with FR235222. Flow cytometry quantification using antibodies against these three proteins revealed that none of them decreased in presence of the inhibitor (Fig. 6A), indicating that HDAC targeting does not result in a general reduction of protein expression.

We next tested whether the elements responsive to FR235222 regulation were located in the non-coding flanking regions of CWP1. To do this, 120 nucleotides of CWP1 5′ UTR flanking region containing the CWP1 promoter (Hehl *et al*., 2000) were cloned in front of recombinant Sar1 fused to a N-terminal HA tag as a reporter, followed by 180 nucleotides of 3′ sequence flanking the CWP1 ORF, containing the poly A addition site (Fig. 6B). Transgenic parasites were obtained by stable integration of the construct into the parasite genome. Similar to the observed inhibition of endogenous CWP1, FR235222 treatment downregulated the expression of the Sar1-HA reporter in engineered parasites (Fig. 6C, D). Drug-mediated inhibition of Sar1-HA expression was also observed in parasites transiently transfected with an analogous construct, which was maintained episomaly (not shown). These results show that the short regions flanking the CWP1 ORF and containing all elements necessary for stage-regulated expression of CWP1 were sufficient for preventing CWP1 induction in response to FR235222 treatment.

FR235222 does not inhibit G. lamblia *replication*

FR235222 treatment was shown to efficiently block the replication of Apicomplexan parasites with an EC_{50} of 10 nM (Bougdour *et al*., 2009). Interestingly, when *G. lamblia* were incubated with FR235222 up to 2 μM, no reduction of replication was observed in encysting parasites and only a modest inhibition in trophozoite cultures (Fig. 7A). This result indicates that the increased levels of histone acetylation induced by FR235222 were not caused by toxicity or dying parasites. Next we tested the sensitivity of the parasite to other HDACi. Neither treatment with apicidin nor with scripaid or HC-toxin up to 2 μM affected parasite replication (data not shown). In contrast, treatment with TSA severely inhibited replication of both parasite stages at nanomolar concentrations; as TSA is a broad range inhibitor of cellular deacetylases (Blagosklonny *et al*., 2002), it is conceivable that the block of replication is due to a non-specific activity towards other essential proteins. Taken together, these results show that treatment with HDACi, which are known to affect replication in other species, often did not have the same inhibitory effect on *G. lamblia* replication.

Given the close association of *Giardia* with the hosts' intestinal epithelial cells, we tested whether FR235222 was detrimental for mammalian cells. Four intestinal and fibroblast cell lines were grown to sub-confluence (Fig. 7B) or confluence (not shown), exposed to the drug, and their metabolic activity was quantified as a measure of cell viability. At both confluencies, 24 h of treatment with 0.02 μM FR235222 (a concentration which blocks *G. lamblia* encystation) did not affect metabolic activity of the intestinal cells and only moderately reduced the activity in the fibroblasts. Higher inhibitor concentrations up to 2 μM induced different responses in different cell types, ranging from no alteration in viability in Caco-2 cells to 50% inhibition in fibroblasts. Moreover, inhibitor-mediated toxicity was not induced during co-culture of intestinal cells with *G. lamblia* (Fig. S3). These results indicate that FR235222 treatment at concentrations inhibitory for *G. lamblia* encystation did not reduce the viability of mammalian intestinal cells.

FR235222 treatment of trophozoites does not induce stage conversion

Our findings revealed that increasing the levels of histone acetylation with FR235222 treatment during encystation blocked stage differentiation in *G. lamblia*. To further investigate the hypothesis that acetylation must decrease to allow differentiation, we evaluated whether pharmacologically increased acetylation in trophozoites would keep the cells at the trophozoite stage or induce parasite differentiation. FR235222 incubation increased histone

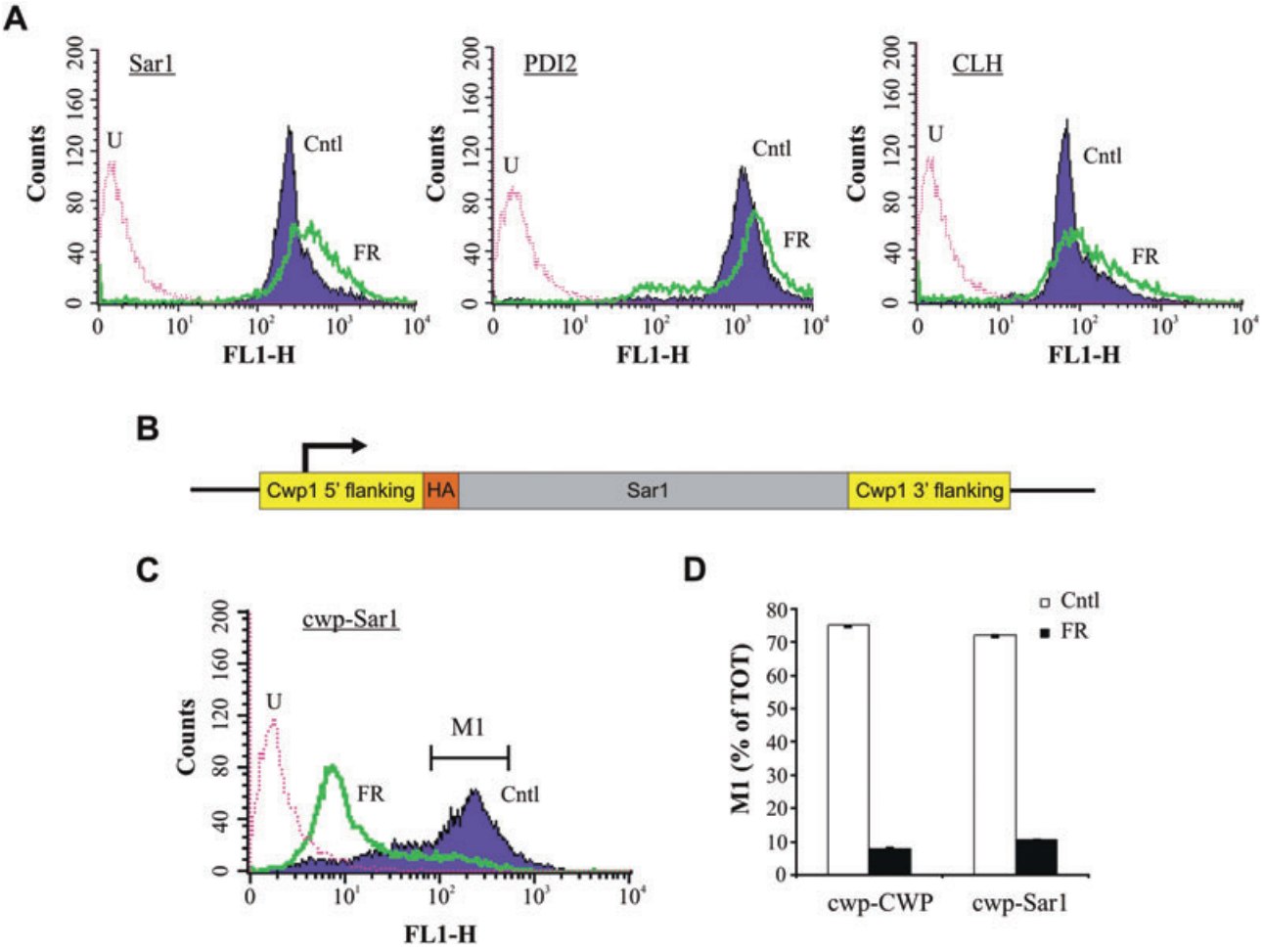

Fig. 6. FR235222 treatment does not reduce the expression of constitutive proteins.
A. Encysting parasites were treated for 24 h with FR235222, as described. Flow cytometry quantification of Sar1, protein disulphide isomerase 2 (PDI2) and clathrin (CLH) showed that the protein expression did not decrease upon inhibitor treatment. Overlay histograms showing cells unstained (U), treated with solvent (Cntl) or 2 μM FR235222 (FR).
B. schematic representation of Sar1-HA construct.
C. Engineered parasites expressing Sar1-HA under control of CWP1 promoter were treated as in (A), stained for recombinant Sar1 using an anti-HA antibody and quantified by flow cytometry. Overlay histogram showing cells unstained (U), treated with solvent (Cntl) or 2 μM FR235222 (FR).
D. The same engineered parasites were additionally probed for endogenous CWP1 expression. The amount of cells with M1 fluorescence is expressed as percentage of total cell number (TOT). Note the decreased expression of both proteins upon inhibitor treatment.

acetylation in trophozoites (Fig. 7C, left panel), although not as potently as in encysting cells (Fig. 4A). Importantly, the degree of tubulin acetylation did not change in the presence of the drug (Fig. 7C, right panel), indicating that FR235222 was effective in specifically modulating the histone acetylation levels in both life stages of the parasite. To test whether HDAC inhibition in trophozoites induced parasite stage differentiation, we quantified the expression of the encystation-induced protein CWP1 in presence of the HDACi FR235222, TSA or apicidin. Flow cytometry analysis (Fig. 7D) and enumeration of CWP1-expressing parasites by manual counting (Fig. 7E and Fig. S4) did not reveal any increase in CWP1 expression in presence of the inhibitors tested compared with control cells. In addition, no giardial 14-3-3 re-localization to the parasite nuclei was detected in the presence of FR235222 (Fig. S5), as is typically observed during parasite encystation (Lalle *et al.*, 2006). Collectively, these data indicate that treatment of trophozoites with HDACi did not promote but rather decreased the rate of spontaneous encystation.

FR235222 alters gene expression in encysting cells and trophozoites

As changes in histone acetylation are known to modulate gene transcription, we used a genome-wide approach to investigate whether FR235222 treatment modified the

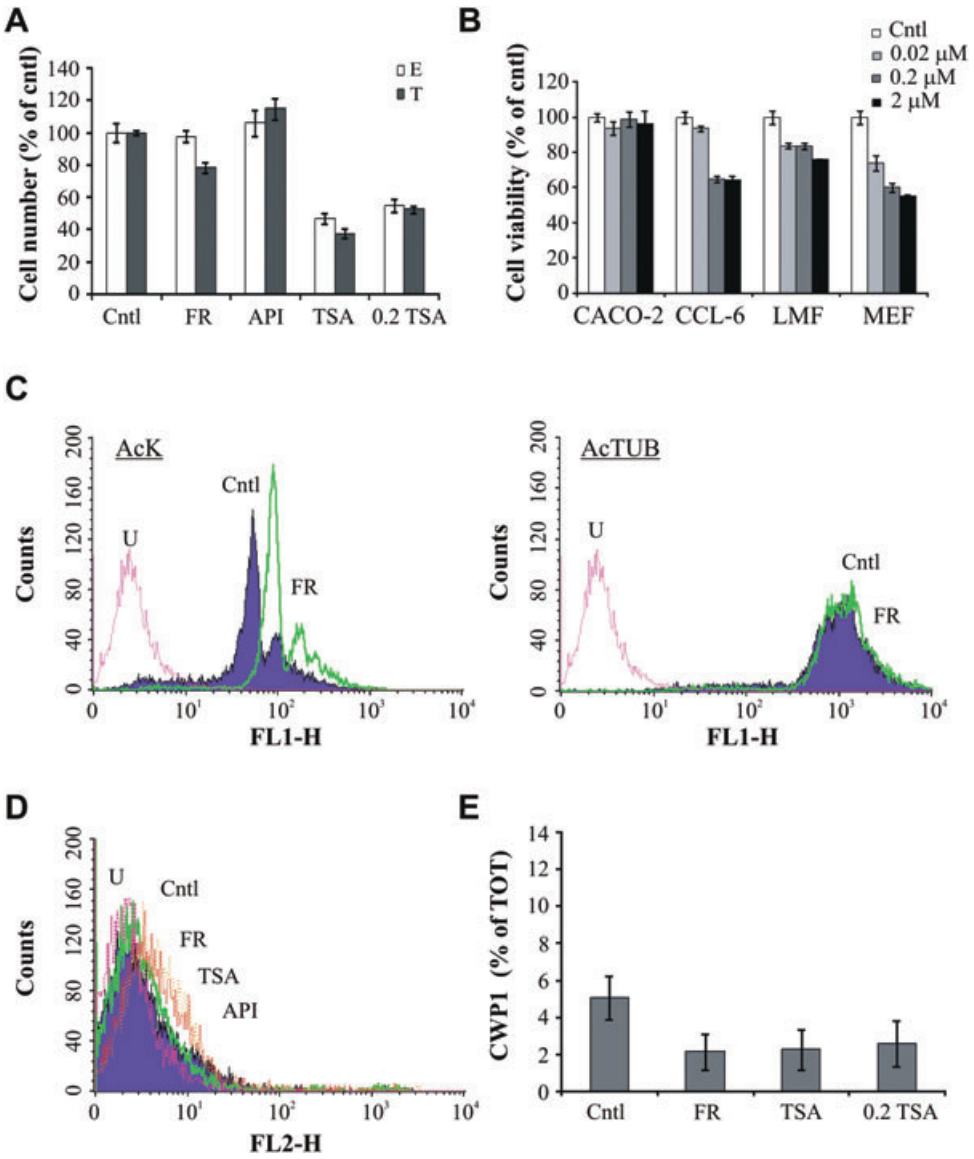

Fig. 7. FR235222 treatment of trophozoites does not induce parasite encystation.
A. Trophozoites (T) and encysting parasites (E) were treated for 24 h with 2 µM of the HDAC inhibitors FR235222 (FR), apicidin (API), Trichostatin A (TSA), 0.2 µM TSA or solvent (Cntl). Parasite numbers are expressed as percentage of untreated samples (Cntl). Data are average ± standard errors ($n = 9$) of a representative experiment done in duplicate.
B. Viability of mammalian cells treated for 24 h with solvent (Cntl) or FR235222 at the indicated concentrations was assessed by measuring AlamarBlue reduction (Biosource). Results of a representative experiment are presented as percentage of control ± standard errors ($n = 3$).
C. Trophozoites were treated for 24 h with FR235222 and levels of AcK (left panel) and acetylated tubulin (right panel) were quantified by flow cytometry. Overlay histograms showing cells unstained (U), treated with solvent (Cntl) or 2 µM FR235222 (FR).
D. Trophozoites were treated for 24 h with 2 µM FR235222 (FR), apicidin (API), Trichostatin A (TSA), or solvent (Cntl) and probed for CWP1. Overlay histogram of the treated cells is shown. U, unstained cells. Note the absence of CWP1 induction upon drug treatment.
E. Cells were treated for 24 h with 2 µM FR235222 (FR), Trichostatin A (TSA), 0.2 µM TSA or solvent (Cntl) and analysed by immunofluorescence. Number of parasites expressing CWP1 was expressed as percentage of the total number of parasites, assessed by nuclear staining, ±standard errors ($n = 6$).

expression of a specific range of genes or induced a general modulation of the expression profile in *G. lamblia*. Transcriptomes of both encysting cells and trophozoites cultured in presence or absence of the inhibitor were compared by microarray analysis. cDNA was labelled with either Cy5-dUTP (FR235222-treated) or Cy3-dUTP (control), hybridized to a *G. lamblia* microarray, and genes whose expression varied by a minimum of twofold following inhibitor treatment were considered significantly regulated ($P < 0.01$).

Overall, 88 genes in encysting cells and 118 genes in trophozoites were found to be differentially regulated by

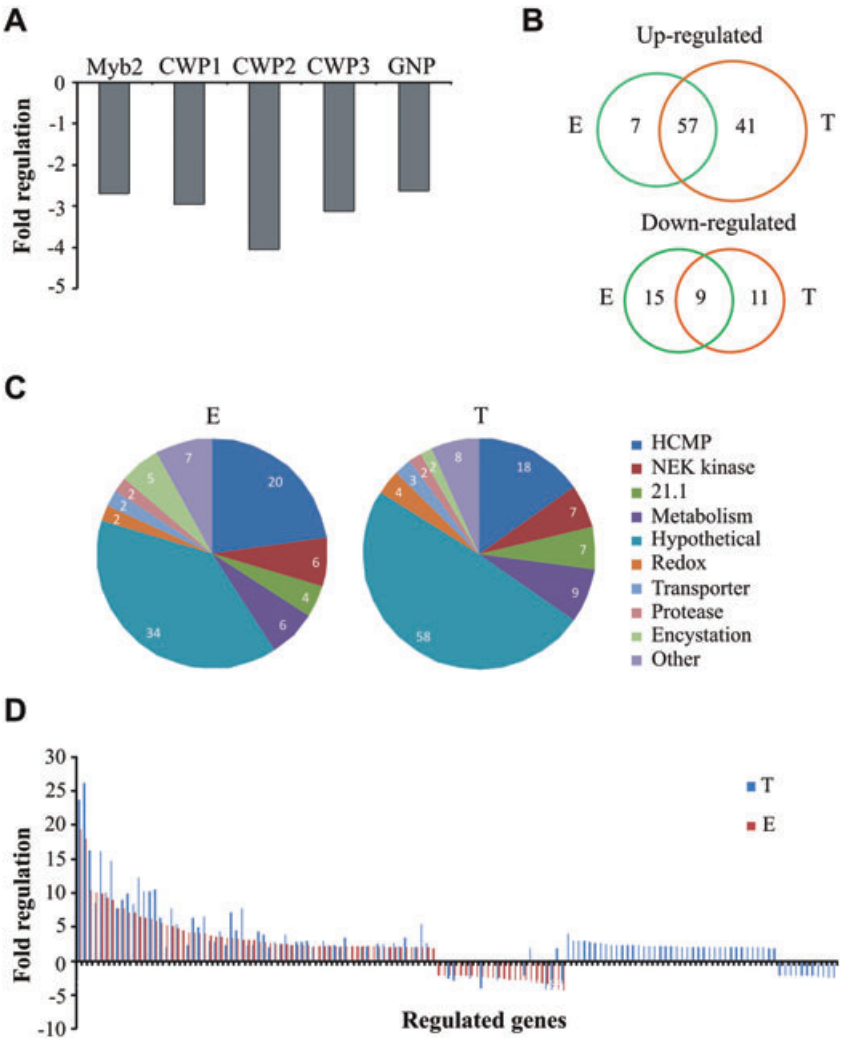

Fig. 8. FR235222 treatment modulates gene expression. RNA extracted from encysting cells and trophozoites treated for 15 h with 2 μM FR235222 or solvent were used for dual channel microarray analysis. The comparison of the transcription profiles of treated versus untreated samples are shown.
A. Drug treatment of encysting cells downregulated the expression of known encystation-specific genes. Names and *Giardia* DB accession numbers are the following: Myb1-like protein, Myb2 (GL50803-8722); cyst wall protein 1, CWP1 (GL50803-5638); cyst wall protein 2, CWP2 (GL50803-5435); cyst wall protein 3, CWP3 (GL50803-2421); glucosamine-6-phosphate deaminase, GNP (GL50803-8245).
B. Venn diagram of genes regulated by FR235222 in encysting cells (E) and trophozoites (T). No prominent overlap of genes regulated by FR235222 was observed in the opposite direction: only two genes downregulated in encysting cells were upregulated in trophozoites and none of the genes upregulated in encysting cells was downregulated in trophozoites.
C. Functional classes defined by Gene Ontology (GO) terms of the FR235222 regulated genes in encysting cells (E) and trophozoites (T).
D. Pattern of gene upregulated and downregulated following FR235222 treatment of encysting cells (E) and trophozoites (T).

FR235222 (Fig. S6), corresponding to ~2% of the 4969 currently predicted *Giardia* genes, and to ~1% of the total 9747 genes, including putative deprecated ones. The majority of the genes were upregulated in both the parasite stages, indicating that HDAC inhibition acted primarily by promoting gene transcription of a selected set of genes. However, in encysting cells treated with FR235222, induction of five genes crucial for parasite encystation was blocked, namely the DNA-binding transcription factor Myb2 (Sun *et al.*, 2002; Huang *et al.*, 2008), the three structural proteins forming the protective cyst wall (CWPs 1–3) and glucosamine-6-phosphate deaminase (GNP, also known as G6PI-B), which is involved in the biosynthesis of the cyst wall polysaccharide (Knodler *et al.*, 1999) (Fig. 8A). These data also confirms that the lack of CWP1 induction in encysting parasites treated with FR235222 is due to reduced mRNA levels.

FR235222 upregulates the expression of identical genes in the two life stages of the parasite

We then compared the transcriptomes of encysting cells and trophozoites to test whether the transcriptional response to inhibitor treatment was similar in the two stages of the *G. lamblia* life cycle. Using Gene Ontology predictions of the *Giardia* Genome database, we grouped the FR235222-regulated genes into functional classes. Figure 8C shows the striking similarity of the gene classes modulated in encysting cells and trophozoites. The family of high cysteine membrane proteins (Davids *et al.*, 2006) was a highly represented class, which also showed the highest level of upregulation. Other highly represented classes were kinases of the NEK family, proteins annotated as 21.1 and proteins involved in metabolic functions. While many of the regulated genes had gene annotations in the *Giardia* Genome database, a considerable number

of the genes indentified in both experimental conditions (38% in encysting cells and 49% in trophozoites) were hypothetical proteins of unknown function.

The set of genes modulated by FR235222 not only belonged to similar gene classes but also overlapped considerably in the two experimental conditions (Fig. 8B and D). Of the 64 genes upregulated in encysting cells, 57 were also found to be upregulated in trophozoites, while of the 24 downregulated genes, 9 were in common with the trophozoite gene list. No correlation was found in the opposite direction: in encysting cells only two of the downregulated genes were upregulated in trophozoites, and none of the upregulated gene was downregulated in trophozoites. Interestingly, all of the high cysteine membrane proteins identified in our array were upregulated in both encysting cells and trophozoites (with one exception in the trophozoites). Similarly, all the NEK kinases and all but one of the 21.1 proteins were upregulated in both parasite stages.

In order to determine whether the genomic environment plays a role in the expression of FR235222-regulated genes, we asked whether regulated genes were clustered. All regulated genes were distributed on 17 genomic scaffolds and a positive correlation was found between the number of regulated genes and the number of genes present on a given scaffold (Fig. S7A). Analysis of the gene position in the genomic scaffolds revealed that the regulated genes were not distributed in tight clusters containing multiple consecutive genes (Fig. S7B); in addition, regulated genes belonging to the same gene classes were not located in the same genomic location, indicating that physical proximity is not required for FR235222-induced transcriptional co-regulation. However, statistical approaches based on graphical and numerical computations revealed small groups of potentially co-regulated genes, which were distributed non-randomly in scaffolds CH991762 and CH991763 in encysting cells, and in scaffold CH991763 in trophozoites (Figs S7C and S8). The biological significance of these findings remains to be determined.

Validation of gene expression regulation

To confirm the expression patterns observed by microarray analysis, we performed RT-PCR of selected genes using RNA extracted from FR235222-treated trophozoites and encysting cells. Specifically, in the trophozoite sample we analysed two genes whose expression was upregulated upon FR235222 treatment (GL50803-114930 and GL50803-112153), while in the encysting cell sample we assessed the abundance of CWP1 transcript, whose expression was downregulated upon inhibitor treatment. Analyses of genes which were not regulated by the inhibitor (GL50803-117204 and GL50803-9558) or not transcribed (tubulin promoter sequence; Elmendorf *et al.*, 2001) were used as a loading and as a negative control respectively. In each case, the abundance of transcripts detected by RT-PCR confirmed the microarray data (Fig. 9A). Importantly, PCR reactions using the original, non-retro-transcribed RNA as template did not give any detectable products, confirming the absence of contamination with genomic DNA (not shown).

In addition, FR235222-induced upregulation of gene expression was further confirmed at the protein level. An N-terminal HA epitope tagged variant of the gene product, GL50803-17120, whose expression increased in both encysting cells and trophozoites upon inhibitor treatment, was expressed in trophozoites. As seen in the RT-PCR results, immunofluorescence and flow cytometry analyses substantiated the increased expression in presence of the inhibitor (Fig. 9B). Collectively, these results unambiguously validate the microarray data both at the transcriptional and translational level.

Discussion

Giardia lamblia encystation is critical for both parasite survival outside the host and disease transmission. Despite the considerable amount of information available on *G. lamblia* encystation at the cellular level, the molecular underpinnings of its regulation remain limited. In the present study, we investigated the role of epigenetic chromatin modification in the parasite stage differentiation. We found that exposure of parasites to the HDACi FR235222 increased the levels of histone acetylation, altered gene transcription and inhibited *G. lamblia* encystation, thus providing the first evidence that epigenetic mechanisms control stage differentiation in this parasite. Notably, FR235222 treatment effectively inhibited CWP1 expression at low nanomolar concentrations, suggesting that the observed phenotype is a consequence of specific inhibition of giardial HDAC. In addition, the fact that only 1–2% of predicted genes are affected and that clear functional categories are identified argues against a random off-target effect.

A highly reduced histone deacetylation machinery is present in G. lamblia

Giardia lamblia has a compact genome organization and few identified regulatory elements, consistent with significant reductive evolution (Morrison *et al.*, 2007). Thus, this protist is considered an interesting model for investigating minimized cellular systems. Analysis of the parasite genome revealed the occurrence of genes coding for putative chromatin modifying proteins, including HATs, deacetylases and methylases, suggesting that mechanisms for epigenetic regulation of gene expression are

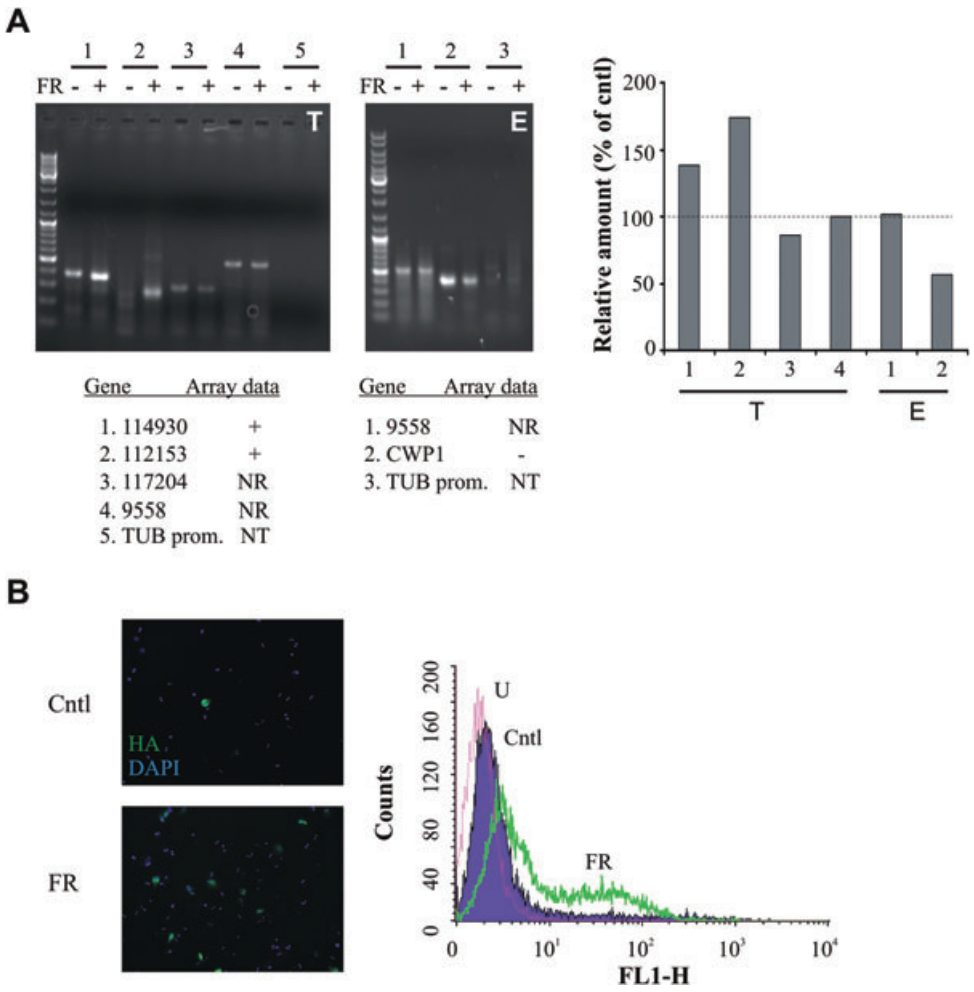

Fig. 9. Confirmation of microarray data.
A. RNA extracted from trophozoites and encysting cells treated for 15 h with 2 μM FR235222 or solvent were tested by reverse transcriptase (RT)-PCR for the transcription of regulated and not regulated gene products. Primer pairs binding to the tubulin (TUB) promoter were used as negative control. The obtained amplification products confirmed the gene regulation observed by microarray analysis. +, upregulated; –, downregulated; NR, not regulated; NT, not transcribed. Right panel, densitometric quantification of the relative amount of the gene products upon FR235222 treatment expressed as percentage of untreated control (Cntl).
B. The gene product GL50803-17120, whose expression was upregulated by 2 μM FR235222 in both encysting cells and trophozoites, was HA-tagged and episomaly expressed under control of its endogenous promoter. Encysting parasites were treated with 2 μM FR235222 (FR) or solvent (Cntl) and the expression of the recombinant protein monitored by immunofluorescence (left panel) and flow cytometry (right panel) analyses. Note the increased expression of the protein upon drug treatment. DAPI, nuclear staining. U, unstained cells.

present in this parasite. Interestingly, demethylases seem to be absent in *G. lamblia*, *Trichomonas*, *Entameba histolytica* and microsporidians, indicating that demetylase activity is dispensable for these parasites or carried out by unrelated enzymes (Iyer *et al*., 2008).

Consistent with its reduced cellular machinery, only one classical HDAC homologue is annotated in the *G. lamblia* genome (GL50803-3281) and its nuclear localization further supports a deacetylase activity on histone proteins. The presence of a single HDAC is in striking contrast with the size of the HDAC repertoire found not only in metazoan but also in unicellular protozoa, including the apicomplexan *T. gondii* with five putative HDACs. It is worth mentioning that a single representative of class I HDACs was identified so far in another enteric parasite, *Entamoeba hystolitica*, suggesting that simplified mechanisms for regulating histone acetylation are operational in basal organisms (Ramakrishnan *et al*., 2004).

Sequence alignments, 3D modelling and prediction of inhibitor binding mode highlighted the high degree of giardial HDAC conservation, in particular at the catalytic site. Moreover, compared with the five *T. gondii* HDACs, giardial HDAC showed the highest degree of similarity with HDAC3, the target of FR235222, further arguing for a specific interaction of the inhibitor with the giardial enzyme.

Of note, the sequence of giardial HDAC contains a four-amino-acid insertion (position 283–286) not found in other species. While amino acid insertions appear to be common in *G. lamblia* proteins, as exemplified by giardial histone acetyl transferase (Morrison *et al.*, 2007), their size is typically larger (20 amino acids on average). The significance of the extra amino acids in the giardial HDAC is unknown.

Histone acetylation regulates stage-specific gene expression during G. lamblia *encystation*

We obtained three lines of evidence indicating that epigenetic mechanisms are involved in regulating expression of encystation-specific genes. First, we observed that the level of histone acetylation decreased during encystation, which is consistent with the parasites differentiating to a dormant stage. Based on this, we can formulate the hypothesis that the high acetylation levels effectively make the parasite refractory to entering differentiation after going through the S-G2 transition of the cell cycle (Bernander *et al.*, 2001). Second, we tested this hypothesis by actively increasing the acetylation levels during encystation. This reduced the synthesis of a relatively small subset of gene products and specifically blocked induction of all known encystation-specific genes. This reveals an as yet unknown level of stage-specific control of encystation-specific genes in addition to the regulation by transcription factors. Importantly, we showed that short-flanking regions of encystation-specific proteins are sufficient for the FR235222-mediated inhibition of gene expression, indicating that no distant regulatory elements are required for modulation of these genes. Third, we demonstrated that histone hyperacetylation altered gene transcription; while the majority of FR235222-responsive genes were upregulated, the mRNA of encystation-specific genes were downregulated. Taken together, our data indicate that chromatin acetylation states play a key role in the regulation of parasite differentiation by controlling the level of gene transcription, possibly by altering the accessibility of the promoter region to Myb2 and other *cis*-acting factors implicated in regulation of encystation-specific genes.

Although we unambiguously showed that increased histone acetylation repressed the transcription of encystation-specific genes, this repression challenges the classical view where histone hyperacetylation is associated with increased gene transcription. Interestingly, inhibition of stage differentiation in response to selected HDACi, TSA and HC-toxin, but not apicidin, has also been reported in *Entamoeba* parasites, but the molecular mechanism still remains to be investigated (Byers *et al.*, 2005).

One plausible explanation is that the repression of encystation-specific gene expression in *G. lamblia* is indirect and occurs via the action of a repressor, which is in turn positively regulated by the increased histone acetylation. The identification of negative *cis*-active elements in the CWP2 promoter (Davis-Hayman *et al.*, 2003) further supports the presence of a repressor mechanism to maintain low levels of encystation-specific proteins during vegetative growth. Block of upregulation of the *trans*-activator transcription factor Myb2 in presence of FR235222 suggests that the putative repressor may act upstream of Myb2 activation.

In other protozoa such as *Toxoplasma gondii* and *Entamoeba histolytica* it has been proposed that HDAC regulation of specific genetic loci is stage-specific, and HDAC inhibition during encystation may derepress trophozoite-specific genes in *E. histolytica*, thus arresting parasite differentiation (Saksouk *et al.*, 2005; Ehrenkaufer *et al.*, 2007). However, our findings suggest in several ways that *G. lamblia* may possess a simpler HDAC regulation, namely (i) only one classical HDAC is found in the parasite genome, arguing against the presence of stage-specific differential HDAC expression; (ii) using the HDACi FR235222, we found increased acetylation in both trophozoites and encysting cells, suggesting that giardial HDAC is indeed operational in both parasite stages; and (iii) contrary to what was observed in *T. gondii* and *E. histolytica* (Ehrenkaufer *et al.*, 2007; Bougdour *et al.*, 2009), HDACi treatment of trophozoites does not trigger parasite stage conversion, suggesting that a trophozoite-specific HDAC engaged in the repression of encystation-specific genes is not present in *G. lamblia*. On the other hand, we showed that the levels of histone acetylation decreased in encysting cells compared with trophozoites. While we cannot exclude that this decrease is due to a reduced activity of histone acetyltransferases, inhibition of HDAC was sufficient to reverse the phenomenon and to block encystation. Hence it is likely that *G. lamblia* does not rely on several stage-specific HDACs but rather on a single HDAC, whose activity is differentially modulated in the parasite life stages, possibly by binding with different stage-specific partners via its C-terminal domain (Pflum *et al.*, 2001). In this context, the regulation of HATs and deacetylases in the different stages of *G. lamblia* is worthy of careful characterization.

Finally, in light of ever-increasing indications that HDACs also function as activators of transcription in yeast (Kurdistani and Grunstein, 2003), we cannot dismiss the possibility that the FR235222-mediated inhibition of encystation-specific gene expression may be a direct consequence of the blocked HDAC activity. Future analyses to determine the acetylation pattern of encystation-specific genes will help to further our understanding of the

epigenetic regulation mechanisms that control stage differentiation in this parasite.

Whole-genome transcriptome regulation upon HDAC inhibition

Our microarray data revealed that FR235222 treatment changed gene expression in both encysting cells and trophozoites. The observed gene regulation is likely to result from a selective inhibition of giardial HDAC and not due to drug-induced lethality, as treated parasites were able to replicate normally in presence of the inhibitor. In addition, analysis of microarray evidence available in the *Giardia* Genome Database revealed that all but three genes regulated by FR235222 treatment were not regulated during stress induction (not shown), indicating that the gene changes of mRNA levels induced by FR235222 do not result from an inhibitor-induced stress response. Finally, the percentage of genes whose expression was changed by FR235222 was quite small (~2%) and of a similar range to what reported for the treatment with the HDACi TCA in mammalian cells (Heller *et al*., 2008) and *E. histolytica* (Ehrenkaufer *et al*., 2007).

Analysis of the modulated genes revealed that, in marked contrast with other cyst-forming parasites *T. gondii* and *E. histolytica*, treatment of *G. lamblia* trophozoites with HDACi did not induce the expression of encystation-specific genes, suggesting that repression of such genes during the vegetative trophozoite stage is not likely to be maintained solely by HDAC activity.

Further comparison of gene expression in encysting cells and trophozoites showed that the genes sensitive to FR235222 were strikingly similar in the two stages of the parasite life cycle. This overlap in expression profile emphasizes that the developmental program is accomplished with only very few changes in the global expression pattern. Besides hypothetical proteins, the major class of upregulated genes was high cysteine membrane proteins (HCMp), a recently described family (Davids *et al*., 2006) whose function in *G. lamblia* remains enigmatic. While we found that the expression of 26% of the known HCMp genes is modulated by FR235222, only ~1% of the previously mentioned VSP family, also composed of cysteine rich proteins, was affected. The result was quite surprising, as histone acetylation of the upstream region of VSP was proposed to positively regulate the expression of the single VSP variant present on the cell surface at any given time, and thus acting as a master regulator of antigenic variation in *G. lamblia* (Kulakova *et al*., 2006). The fact that inducing histone hyperacetylation did not result in a general increase in VSP expression suggests that the epigenetic histone modification by the acetylation mark may not be sufficient to promote VSP transcription, and that additional mechanisms are required to regulate the expression of these proteins. Alternatively, the use of different *G. lamblia* isolates (WB, prototype of Assemblage A in our study, and GS, prototype of Assemblage B in Kulakova *et al*., 2006) might also account for some differences in gene regulation, as the two isolates present different phenotypes *in vitro* and *in vivo* (Franzen *et al*., 2009; Monis *et al*., 2009).

The other two protein families whose expression was modulated by FR235222 were cytoskeletal proteins 21.1 and members of the NIMA-related protein kinases (NEK) (Morrison *et al*., 2007). The latter are widely represented in eukaryotes, but their role remains poorly characterized. Interestingly, the NEK family shows a significant expansion in *G. lamblia*, where it constitutes the majority of the parasite kinome (Morrison *et al*., 2007). The significance of this expansion is currently unknown.

Finally, genes of the proteins and protein families significantly affected by FR235222 treatment were scattered in different scaffolds of the parasite genome, indicating that HDAC inhibition can modulate the expression of functionally related genes independent of their genomic location. It is worth noting that statistical approaches identified three examples of non-randomly distributed gene sets in both encysting cell and trophozoite transcriptomes. Interestingly, the cluster found in trophozoites contained the CWP1 gene surrounded by four upregulated genes. As CWP1 mRNA was found to be slightly upregulated in our trophozoite array without detecting parasite stage differentiation, it is possible that the CWP1 genomic surrounding may have influenced CWP1 transcription without activation of the complete encystation program.

In conclusion, we showed, for the first time, that HDAC inhibition increased histone acetylation and induced transcriptional changes in *G. lamblia*, substantiating the presence of an evolutionarily conserved mechanism for epigenetic regulation of gene expression in this early divergent parasite. In addition, we provided evidence that the HDAC repertoire is highly reduced and the phenotypic and transcriptional responses following HDAC inhibition are simpler in *G. lamblia* than in other cyst-forming parasites, indicative of the minimized cellular processes typical of this parasite. Lastly, we discovered that HDAC inhibition, while not affecting parasite replication, potently blocked *G. lamblia* encystation and that short-flanking regions of encystation-specific proteins were sufficient for the drug-mediated repression of gene expression. Collectively, these results reveal that the transcriptional changes during stage differentiation are under the control of epigenetic regulation, and further illuminate the poorly understood mechanisms of gene regulation in this parasite. Additionally, our findings clearly demonstrate that HDAC activity is a promising target for pharmacological agents aiming to effectively block the production of *G. lamblia* cysts and thus reduce disease transmission.

Experimental procedures

Biochemical reagents

Unless otherwise stated, all chemicals were purchased from Sigma and cell culture reagents from Gibco-BRL. Inhibitor stock solutions were prepared at the following concentrations: 179 mM FR235222, 1.6 mM apicidin, 1 mM TSA, 2.29 mM HC-toxin, 3.06 mM scripaid. Inhibitors were freshly diluted to the concentrations required for the individual experiment.

Parasite and tissue culture

Trophozoites of the *Giardia lamblia* strain WBC6 (ATCC catalog number 50803) were grown axenically as described (Sonda *et al*., 2008). Harvested parasites were counted using the improved Neubauer chamber. New subcultures were obtained by inoculating 5×10^4 trophozoites from confluent cultures into new 11 ml culture tubes. Two-step encystation was induced as described previously (Gillin *et al*., 1989) by cultivating the cells for ~44 h in medium without bile (pre-encysting medium) and subsequently in medium with higher pH and porcine bile (encysting medium).

Drug treatment of trophozoites was performed by incubating sub-confluent cultures for 24 h with the inhibitors at the concentrations indicated in the figure legend of the individual experiments. Drug treatment of encysting cells was performed in two steps: 8 h drug incubation in pre-encysting medium and additional 16 h incubation in encysting medium. Viability of cysts following drug treatment was tested by trypan blue exclusion (Aley *et al*., 1994).

Mammalian cells used in this study were: Caco-2 (human colon adenocarcinoma, ATCC HTB 37), Intestine 407 (human embryonic jejunoileum, ATCC CCL-6), primary lung fibroblasts isolated from 6- to 8-week-old mice kindly provided by E. Gulbins (University of Duisburg-Essen, Essen, Germany) and mouse embryonic fibroblasts kindly provided by A. Schinkel (the Netherlands Cancer Institute, Amsterdam, The Nederlands). Cells were routinely cultured in Dulbecco's modified Eagle's medium or minimum essential media supplemented with non-essential amino acids and sodium pyruvate, in case of primary fibroblasts. All media were supplemented with 10% fetal calf serum, 2 mM glutamine, 50 U of penicillin ml^{-1}, and 50 μg of streptomycin ml^{-1}. Cultures were maintained at 37°C with 5% CO_2 in tissue culture flasks and trypsinized at least once a week.

Expression vector construction and transfection

The plasmid for episomal expression of haemagglutinin (HA)-tagged GlHDAC (GL50803-3281) under control of CWP1 promoter was based on the expression cassette C1-CWP (Hehl *et al*., 2000). The plasmid for stable expression of HA-tagged Sar1 under control of CWP1 promoter was engineered as described (Stefanic *et al*., 2009). The plasmid for the episomal expression of HA-tagged GL50803-17120 was based on the expression cassette C1-CWP where the CWP1 promoter was replaced by the endogenous promoter. Oligonucleotides (5′–3′ orientation) used for the construct were the following: for GL50803-3281, PstI-s CGCTGCAGATGCCGC CTTCAAAACCCTC and HA-PacI-as CGTTAATTAACTACG CGTAGTCTGGGACATCGTATGGGTAGTTCTCATCTAACC CCGCTTC; for GL50803-17120, XbaI-s CGTCTAGACTAC TATGGATGAAGCCGAG and HA-NsiI-as CGATGCATC GCGTAGTCTGGGACATCGTATGGGTAAATCGTGATGTTG TTAGTTAAAC, encoding the HA epitope tag for the amplification of 200 bp upstream the starting codon containing the endogenous promoter followed by the protein leader sequence; 17120-NsiI-s CGATGCATGGTGTCCGCGAAG GAAAATATG and 17120-PacI-as CGTTAATTAACTAGC TATAAATGGCTGCAATG for the amplification of the GL50803-17120 coding sequence.

15 μg of plasmid vector DNA was electroporated (350 V, 960 μF, 800 Ω) into trophozoites. For stable genome integration, the plasmid DNA was linearized using SwaI restriction enzyme before electroporation. Linearized plasmid targets the *G. lamblia* triose phosphate isomerase locus (GL50803-93938) and integration occurs by homologous recombination under selective pressure with the antibiotic puromycin (Jimenez-garcia *et al*., 2008).

Gene expression analysis

Trophozoites were treated for 15 h with 2 μM FR235222 or solvent; encysting cells were similarly treated in two steps: 8 h drug incubation in pre-encysting medium and additional 7 h incubation in encysting medium. RNA was isolated using an RNAeasy kit (Qiagen, Stanford, CA) following the 'Animal Cells Spin' protocol. Residual genomic DNA was removed with DNase 1 digestion according to the manufacturer's protocol. The integrity of the RNA was analysed in a Bioanalyser (Agilent Technologies, Palo Alto, CA, USA) with 'Eukaryote Total RNA Nano Series II' settings.

For dual channel microarray analysis, extracted total RNA was processed using the Amino Allyl MessageAmpII a RNA Amplification Kit (Ambion, Austin, TX, USA) and labelled with *N*-hydroxysuccinimidyl ester-derivatized reactive dyes Cy3 or Cy5, according to the manufacturer's protocol. After purification, 2 μg each of Cy3 or Cy5 labelled aRNA was denatured, added to SlideHyb Buffer I (Ambion), and hybridized to *G. lamblia* microarrays version 2 (TIGR) in a Tecan HybStation at the Functional Genomics Centre Zurich, Switzerland. The arrays are aminosilane surface coated glass slides with 9115 oligonucleotides (70mers) designed to cover the whole *G. lamblia* WBC6 strain genome.

Prior to hybridization, slides were hydrated and blocked with 150 μl BSA buffer (0.1 mg ml^{-1} BSA, 0.1%SDS in 3× SSC buffer) for 1 h at 42°C. After washing, samples were injected and hybridized for 16 h at 42°C. Slides were scanned in an Agilent Scanner G2565AA, using laser lines 543 nm and 633 nm for excitation of Cy3 and Cy5 respectively. Spatial scanning resolution was 10 μm, single pass. The scanner output files were quantified using the Genespotter Software (MicroDiscovery GmbH, Berlin, Germany) with default settings and 2.5 μm radius. The median spot intensities were evaluated with the Web application MAGMA (Rehrauer *et al*., 2007) and normalized using the print-tip-wise loess correction of the *limma* package (Smith, 2005). Potential gene-specific dye-effects were estimated from self–self hybridizations. Differential expression of genes during

FR235222 treatment is reported as the $\log_2$ of the drug-induced fold-change compared with control treated samples, as well as the *P*-value for differential expression as estimated by the empirical Bayes model implemented in *limma*. All reactions were performed in triplicate.

Semi-quantitative reverse transcriptase polymerase chain reaction

300 ng of the isolated RNA described above was mixed with 10 μM k-anchorV primer and cDNA was synthesized using the Quiagen Omniscript Kit (Qiagen, Stanford, CA, USA) according to the manufacturer's protocol. Fifty-fold dilutions of cDNA were used as template for PCR amplification using 0.4 μM each of gene-specific forward primers and a k-adaptor reverse primer. PCR products were separated on 1.5% agarose gels, visualized with ethidium bromide, and images recorded in a MultiImage Light Cabinet with AlphaEaseFC software (Alpha Innotech, San Leonardo, CA, USA). Primers used in the study are: k-anchorV, CCGGAATT CGGTACCTCTAGA(T18)V; k-adaptor, CCGGAATTCGGTAC CTCTAGA; Tubulin, ATTTAGAATTCAAATCAGCAAATTC; 114930, GCCAGGGCCTCATCGAAC; 117204, ATACGTGG GGGTGGAGAAC; 112135, CGTCCTCTGCTACTCCTTTG; 9558, CGGACCATTACTTCTACGTACT; CWP1, CTGGTA CATGAGTGACAACGCT.

Fluorescence microscopy and flow cytometry analysis

For immunolabelling, harvested cells were washed twice in ice-cold PBS, and fixed with 3% formaldehyde solution in PBS. Fixed cells were blocked, with or without previous permeabilization with 0.2% Triton X-100 in PBS for 20 min, and incubated with primary antibodies for 1 h. The primary antibodies used in this study were: anti-acetyl lysine rabbit antiserum (Abcam, Cambridge, MA, USA), 1:500 dilution; anti-acetylated tubulin (Sigma), 1:500 dilution; Cy3-conjugated anti-CWP1 mouse monoclonal antibody (Waterborne, New Orleans, LA, USA), 1:60 dilution; anti-clathrin heavy chain mouse antiserum (Marti *et al*., 2003), 1:2000 dilution; anti-protein disulphide isomerase 2 (PDI2) mouse antiserum, 1:1000 dilution; anti-Sar1 mouse antiserum (Stefanic *et al*., 2009), 1:300 dilution; Alexa488-conjugated anti-HA mouse monoclonal antibody (Roche Diagnostics GmbH, Manheim, Germany) 1:30 dilution; anti-giardial 14-3-3 (Lalle *et al*., 2006) 1:50 dilution, kindly provided by M. Lalle (Istituto Superiore di Sanita', Rome, Italy). Fluorophore-conjugated secondary antibodies were purchased from Invitrogen (Basel, Switzerland) and used at 1:200 dilution. Microscopy analyses were performed on a Leica DM IRBE fluorescence microscope (Leica Microsystems, Wetzlar, Germany) using the appropriate settings. Single-cell quantification of fluorescence was performed using a FACSCalibur flow cytometer (Becton & Dickinson, Basel, Switzerland).

Mammalian cells metabolic assay

The metabolic activity of mammalian cells was tested by using the AlamarBlue assay (Biosource, Camarillo, CA, USA). Briefly, mammalian cells were grown to confluence or semi-confluence in 96-well plates, incubated for 24 h with FR235222 at the concentrations indicated in the figure legend, and processed according to the manufacturer's instructions. For co-culture experiments, cells were seeded in 96-well plates at 3×10^4 cells per well and incubated for 24 h to confluence. Harvested *G. lamblia* trophozoites were added to the cell monolayers at 6×10^4 parasites per well, followed by 24 h incubation with 2 μM FR235222 and viability assay.

Western blot analysis of nuclear proteins

Encysting cells were treated with 2 μM FR235222 as described before. Nuclear extracts were prepared by mild detergent lysis as described (Bougdour *et al*., 2009). Briefly, 6×10^7 cells were collected, washed in ice-cold PBS and resuspended in 700 μl lysis buffer [10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.5% NP-40, 1 mM PMSF and protease inhibitor cocktail]. The nuclear pellet was collected by centrifugation at 10 000 *g* for 10 min at 4°C and acid extracted for 2 h by adding 200 μl each of 5 M $MgCl_2$ and 0.8 M HCl. Insoluble material was removed by centrifuging at 13 000 *g* for 10 min at 4°C and soluble proteins containing basic histones were precipitated with 25% TCA. Precipitated proteins were washed twice with ice-cold acetone and resuspended in 100 μl H_2O. Aliquots corresponding to 16 μg of proteins were analysed by SDS-PAGE on a 15% gel followed by Coomassie blue staining and probed, following Western blotting, using the previously described anti-acetyl lysine rabbit antiserum (Abcam) at 1:2000 dilution. Immunoreactive bands were visualized with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

Determination of protein concentration

Protein content was determined using the Bio-Rad Protein Assay according to the instructions provided by the manufacturer. Bovine serum albumin was used for the standard curve.

Mass spectrometry identification of histones

Trophozoites were treated with 2 μM FR235222 for 24 h, harvested, washed in ice-cold PBS. 3×10^7 cells were then resuspended in 500 μl lysis buffer (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5% NP-40, 1 mM PMSF and protease inhibitor cocktail), incubated for 30 min at 4°C, and the nuclear pellet was collected by centrifugation at 10 000 *g* for 10 min at 4°C. The pellet was resuspended in 5 ml IP buffer (10 mM HEPES, 10% glycerol, 5 mM EDTA, 1% NP-40, 1 mM PMSF), sonicated and incubated with 5 μl anti-acetyl lysine rabbit antiserum overnight at 4°C, followed by addition of protein-A conjugated beads. After incubation at 4°C under rotary agitation for 5 h, the beads were washed, resuspended in 30 μl sample buffer, boiled and treated with 3.3 μl iodoacetamide (0.5 M in 1 M Tris-HCl pH 8.0) for 15 min at room temperature. Proteins were separated on a 15 % gel, stained using the mass spectrometry-compatible

SilverQuest™ silver staining Kit (Invitrogen) and the excised bands analysed by peptide mass fingerprint using LC/MS/MS.

Protein structure prediction

The SWISS-MODEL web service (Guex and Peitsch, 1997; Schwede *et al*., 2003; Arnold *et al*., 2006) was used to build 3D models of GlHDAC and HsHDAC8. Docking of FR235222 in the catalytic site of GlHDAC was performed using the program WITNOTP (Armin Widmer, Novartis Pharma, Basel). Alignments were performed with Multalin (http://bioinfo.genotoul.fr/multalin/) and sequence comparisons were performed using Blast2.

Bioinformatic analyses

For visual identification of cluster candidates, we defined for each scaffold a metric M_f based on a potential function f analogous to the gravitational potential of mass points. In addition to a constant threshold, we used a linear combination $\mu_{pot} + \lambda s_{pot}^{+}$, based on mean μ_{pot} and conditional standard deviation s_{pot}^{+} of all potential fields generated by all permutations of gene distributions. The metric M_f was used to calculate the cluster hierarchy on all levels by complete linkage. All clusters were ranked according to the calculated probability $p_{cluster}$, based on hypergeometric distributions. For the identification of statistically evident clusters, we defined approximate permutation tests based on the Null-hypothesis H_0 of random and uniform distribution of regulated genes within the scaffolds, and the three following test statistics: mean deviation X_1 and standard deviation X_2 of the number of not-regulated genes between two regulated genes, and maximum number of consecutive short intervals ($\leq k$) of non-regulated genes $X_{3,k}$ to account for the cluster size. The distributions of these test statistics were approximated by Monte Carlo sampling 100 000 random permutations for each scaffold. The functions used in this study were: $f(x)_{x \neq xi} := \Sigma_{(i=1,\ldots,n)} 1/(x - x_i)$, $f(x_i) := c << 0$; $M_f(x,y) := \max_{z \in [x,y]}(|f(x) - f(z)|)$; $f_{\boldsymbol{b}}(x)_{bx \neq 1} := \Sigma_{(i=1,\ldots,d)} b_i/(x - i)$, $f_{\boldsymbol{b}}(x)_{bx=1} := c << 0$; $\mu_{pot}(x) := (\Sigma_{\boldsymbol{p} \in \boldsymbol{P}} f_{\boldsymbol{p}}(x))/|\boldsymbol{P}|$; $(s_{pot}^{+})^2 := (\Sigma_{\boldsymbol{Q} = \{\boldsymbol{p} | \boldsymbol{p} \in \boldsymbol{P} \wedge f_{\boldsymbol{p}}(x) > 0\}} (f_{\boldsymbol{p}}(x) - \mu_{pot}(x))^2)/|\boldsymbol{Q}|$; $p_{\text{ClusterC}a,b,r} := \Pr\{Y_{b\text{-}a\text{-}2,n,d} = r - 2\} \Pr\{Y_{2,2,n\text{-}(b\text{-}a\text{-}2)} = 2\}$, where d = number of genes of a scaffold, n = number of regulated genes in a scaffold, $x_1 < x_2 < \ldots < x_n$, $1 \leq x_i \leq d$ the positions (consecutive numbers) of the regulated genes, $\boldsymbol{b} = (b_1, b_2, \ldots, b_d)$, $b_i = \text{if}(i \neq x_i, 0, 1)$, $\boldsymbol{P}$ = set of all permutations of $\boldsymbol{b}$, $Y_{m,s,t}$ a hypergeometric random variable as m draws without replacement from a population of t genes, thereof s regulated, $C_{a,b,r}$ a cluster $\{a = x_1 < \ldots < b = x_i\}$ with r elements.

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Part IV: Discussion & Perspectives

1. Discussion

1. 1 General

Protozoan parasites present a large health problem worldwide and cause severe morbidity and mortality. Despite many unique cellular and biochemical features, finding good drug targets remains challenging. To understand the basic biology of the organism through molecular cell biological and comparative genomic approaches can provide clues to help identify vaccine candidates and therapeutic targets. In addition, because the cell biology of parasites is the product of long co-evolution with its host it reflects a wide range of adaptive processes and biological diversity. Regardless of the *G. lamblia* phylogenetic status as either an early-branching eukaryote [1,2] or a highly simplified cell [3], its simple cellular organization can provide us with fundamental insights into the evolution and function of cell compartmentalization, in particular with respect to highly modified organelles such as the Golgi (see model in 1.2 below) or mitosomes.

Differentiation of *G. lamblia* trophozoites to cysts is critical for survival of the parasite outside the host and for its transmission. In our *in vitro* culture system, Giardia undergoes stage conversion in 18-24 h. During stage conversion the motile trophozoite undergoes significant morphological changes; it loses its flagella and adhesive disk and becomes encased in an extracellular matrix, the cyst wall. The extracellular stimulus (in cultured parasites this is achieved by changes in the medium) induces changes in parasite metabolism, concerted upregulation of encystation-specific genes, and *de novo* genesis of Golgi-like encystations-specific vesicles (ESVs) for accumulation, maturation and secretion of cyst wall material.

During my PhD I worked on several aspects of Giardia stage differentiation. I started out on a project to analyze the function of conserved small monomeric GTPases in the secretory pathway of Giardia during stage conversion, and to investigate their role in ESV and cyst formation. An important question was whether and how much of the organization of the minimized secretory pathway of Giardia was conserved. In my second project, I wanted to determine the exact scope of the transcriptional response to the encystation stimulus. To do this I used whole transcriptome analysis to test our hypothesis that only a limited number (i.e. <50) of genes are regulated during encystation. Finally, in my last project, I investigated the role of histone acetylation during parasite stage differentiation. I used a histone deacetylase inhibitor (FR325222) as a tool to interfere with chromatin remodeling processes during Giardia stage differentiation.

1.2 Small monomeric GTPases have a role in ESV and cyst formation.

In most cells, the Golgi, which is at the center of the secretory system, is a complex cellular machinery of closely stacked and highly dynamic but steady state cisternae [4]. Because of this complexity and the many factors involved in its organization, it is difficult to address apparently simple questions, such as how cargo moves from the ER to the Golgi and then through the stack. Thus, researchers have sometimes used simpler unicellular organisms as model systems to investigate certain aspects such as, for example how the Golgi is inherited during cell division [5] or how cargo moves through cisternae [4]. Giardia as a unicellular model organisms whose trafficking machinery is basic and/or has undergone massive reductive evolution, allows investigation of many of these basic functions in a highly simplified context.

During encystation, cyst wall material is delayed in ESVs for maturation until regulated secretion. ESVs have been shown to have basic Golgi characteristics, and likely correspond to *cis*- Golgi-like cisternae (see introduction) [6-9]. ESVs have several advantages for the cell biologist compared with a classical Golgi. First, individual ESVs are large, easy tractable and can be resolved by light microscopy. This allows study of the dynamics of cargo and compartments in living cells expressing fluorescent markers for example. Second, ESVs are not steady state organelles but arise *de novo*, i.e. they are generated in response to synthesis and export of a large amount of CWM [10] from the ER. Third, unlike the classical Golgi, ESVs harbor only one type of cargo, which is sorted away from constitutive proteins before its accumulation in ESVs [10]. All of this material is exported to the plasma membrane in a coordinated and time-dependent manner, allowing cargo maturation, processing and most probably quality control before secretion ([8,11] Konrad *et al*. PLoS Pathogens, in press). Fourth, the Giardia secretory system is divergent and minimized, but has some highly conserved components [8]. Investigation of basic trafficking processes in particular provides insights into the minimal requirements for the generation of stable delay compartments. Thus, even though this is a reduced system and not a primary basic one, its organization might reflect similar principles, capacities and limitations as the secretory apparatus of an ancestral eukaryote prior to the evolution of the Golgi.

In higher eukaryotes, the small GTPases Sar1, Rab1 and Arf1 act as molecular switches in specific steps in the ER to Golgi secretory pathway. *In silico* analysis has revealed highly conserved homologs of Rab1, Arf1 and Sar1 in Giardia [8,9]. An important question in the first study of my PhD work was whether the coat protein complexes COPII and COPI and therefore associated GTPases Sar1 and Arf1 are conserved in function, and if they are required for ESV formation, maturation and finally cargo secretion. The question arose because some researchers postulated that ESVs could be ER subcompartments in which the CWM accumulates before it is secreted by direct fusion with the plasma membrane. If we could show dependence of CWM export on sequential action of Sar1 and Arf1, this would strengthen the argument that the CWM is exported to post ER compartments for maturation before secretion. A simple, but technically challenging, approach was to interfere with these GTPases specifically during encystation. Because gene knockouts are not possible in the binucleate Giardia, we used dominant-negative mutants of the small GTPases Sar1, Rab1 and Arf1 for functional analysis. Analogous to higher eukaryotes, Rab1 and Sar1 are both expected to act in early events in the ER- to Golgi secretory pathway [4]. Here, we showed that interference with Sar1 inhibited ESV formation, consistent with analogous functional analysis of Sar1 in the early secretory pathway in other systems , such as in mammalian cells or in yeast [4,12,13]. Functional analysis of mutant Rab1 variants under the control of an encystation-specific expression system, or stage-specific Rab1 mRNA ablation revealed a sporadic, but consistent phenotype of encysted trophozoites with dispersed ESVs and cyst forms that lack CWP on the surface. Timing of expression is a possible explanation for the sporadic phenotype: Rab1 requires post-translational modification (prenylation) which delay the availability of significantly amounts of the dominant-negative Rab1 in early encystation because we have to use stage-specific induction. An encystation independent, inducible expression system exists, but it is very inefficient and not sufficiently reliable for use in this case.

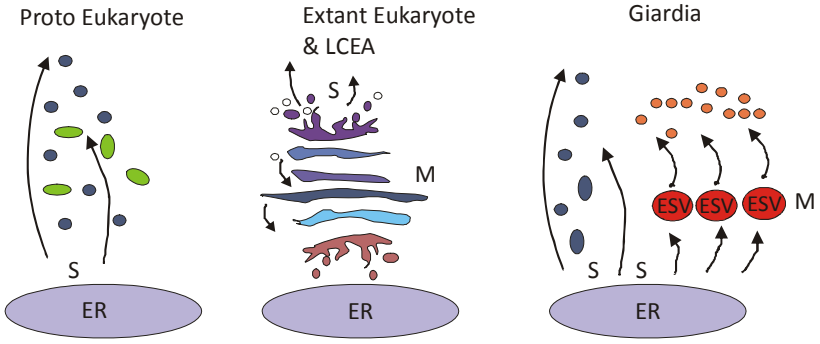
Arf1 acts as a key regulator of COPI and AP1/clathrin membrane coats at Golgi membranes in higher eukaryotes [14]. Consistent with this normal function of Arf1, expression of a GTP-locked Arf1 variant in differentiating Giardia resulted in a block of CWM secretion. This suggests that Arf1 has an essential role in transport steps between completed ESVs and exocytosis of CWMs at 20-24h.

Taken together, we showed that the functional requirements for ESV formation are surprisingly conserved despite fundamental differences in compartment and organelle organization. This is additional support for our hypothesis that ESVs are *cis*-Golgi analogs. These findings are important because they allow addressing basic questions of the highly reduced trafficking machinery with implications for higher eukaryotes.

We also addressed the question how maturation and secretion of the CWM is coordinated among the ~30 ESVs. The CWM is sequestered for maturation and processing in fully developed ESVs before secretion to the plasma membrane. Secretion and polymerization of the cyst wall appears to occur in only a few minutes. This requires coordination and cargo quality control between the ESVs, presumably by allowing cargo to distribute inside an ESV network structure. Our data revealed possible tubular structures as physical links for cargo exchange inside an ESV organelle network. Structurally and functionally this is highly reminiscent of a Golgi ribbon in mammalian cells. This higher order structure provides lateral connections between homologous cisternae and facilitates cargo maturation [15,16]. In the case of ESVs it is not clear where the connecting membrane tubules originate. In analogy to the Golgi ribbon one could postulate a machinery which generates these structures as outgrowths from ESV membranes. This would require homotypic fusion events to connect ESVs. An alternative possibility is exchange of cargo via ER-derived tubular domains that connect ESVs and persists throughout encystation.

1.3 Giardia as a model system for understanding the evolution of the Golgi apparatus

Comparative genomics and phylogenetic analyses indicate that a stacked Golgi and complex endocytic and exocytic trafficking pathways were already present in a hypothetical last common eukaryotic ancestor (LCEA) [3]. In contrast, the secretory apparatus of the proto-eukaryote lacked maturation compartments and was likely only capable of directly exporting proteins from the ER lumen to the plasma membrane via transport vesicles generated by coat protein complexes. From this simple machinery, the Golgi may have evolved in several steps. First, cells acquired the capability to sort different proteins to different transport vesicles, which were still rapidly exported without the possibility of addition of complex modifications to the cargo. The development of a primitive delay organelle was a key step in eukaryotic evolution and required the capability of homotypic fusion of transport vesicles. This results in larger compartments in which the cargo and associated modifying enzymes could be accumulated for a while together with modifying enzymes to allow more sophisticated processing. This simple delay compartment which is essentially an outgrowth of the ER became more complex with development of biochemically distinct cisternae arrayed in sequence and sorting functions. Giardia is a good model to study this hypothetical evolutionary process because it lacks a stacked Golgi, but generates compartments for post translational maturation of cargo proteins transiently in the form of ESVs. Thus, the giardial trafficking system resembles an early stage of Golgi evolution, before post ER cisternae became sufficiently stable to form a constitutive organelle in the LCEA.



Model 1: Evolution of the Golgi apparatus – Giardia as a model
left: a hypothetical proto-eukaryote, middle: extand eukaryote, right: Giardia
M: maturation, S: sorting, ESV: encystation specific vesicle, ER: endoplasmatic reticulum

1.4 A small set of genes is regulated during early stage conversion

Protozoan parasites have evolved complex life cycles with distinct developmental stages, which may involve multiple host organisms and strategies to evade host immune responses. Stage conversion events are usually linked to alterations in the parasite transcriptome as for example, in Entamoeba, whose life cycle is similar to that of Giardia [17,18]. The Giardia genome is compact, and rapid transcriptional changes in response to environmental triggers are possible (Spycher *et al*. unpublished).

In response to induction of encystation the three CWPs were shown to be transcriptionally upregulated as well as a Myb2-like transcription factor (~2-3h p.i.). A conserved *cis*-acting element in the promoter region of all of those genes was described to which the putative Myb2 transcription factor binds (see Introduction 2.3 [19]). Relatively late in encystation (~12h p.i.) upregulation of a HCNPp protein [20], possibly involved in the linkage of the CW to the plasma membrane, and of five key enzymes of the UDP-GalNAc synthesis pathway from glucose required for the cyst wall formation was shown [21]. However, as mentioned above the triggers of encystation *in vivo* are unknown. All of those experimental approaches to identify encystation-specific genes, including a system-wide serial analysis of gene expression (SAGE) published on the Giardia DB, were performed *in vitro* by applying mainly one protocol for induction. None of the studies include the possibility to differentiate between expression of real encystation-specific genes and side effects (off-target effects) of the applied protocol. Thus far an *in vivo* approach to investigate the transcriptional response to encystation in the intestine of a host is technically not possible, so the real complement of regulated genes that are directly involved in differentiation is unknown.

In my second project, I analyzed the system-wide transcriptional response to encystation. Importantly, for the first time, two *in vitro* encystation protocols for induction of differentiation with comparable kinetics and efficiency were applied to consider possible off target effects.

Transcriptional monitoring of three time points in the first 7h p.i. of encystation revealed a quantitatively limited (49 different genes in all three time points (45min, 3h, 7h p.i.)), bipartite transcriptional response. This limited amount of genes fits the idea that encystation requires relatively few changes in gene expression patterns since the cyst wall appears to have only few components that must be newly synthesized (CWP1-3, β (1-3)-GalNAc homopolymer). Many key factors of the secretory pathway (Rab1, Sar1, Arf1) are constitutively expressed, and Giardia overall possesses a rather small genome (~12Mbp [22]) and is characterized as a highly reduced eukaryotic cell. The comparison of two *in vitro* protocols for induction of encystation at 7h p.i., where the mRNA of structural proteins (CWP1-3) were shown to peak, reveals a small core set of 18 *bona fide* encystation genes containing the known factors such as CWP1-3 and Myb2. Importantly, we found a higher number of protocol-specific than intersecting genes in the two data sets. This comparative analysis highlighted the importance to distinguish between protocol-specific off-target effects and the real encystation-specific transcriptional response required to accomplish cyst formation by the parasite. This is especially relevant when large data sets are posted in publically accessible repositories (as the SAGE data on en-/excystation) such as the Giardia DB. Importantly, the knowledge about the exact response to induction of encystation might reveal valuable drug targets to prevent cyst formation in the host and therefore spreading of disease. In addition, analysis and comparison of different *in vitro* encystation protocols will helpnarrow down where and how exactly encystation is triggered in the host intestine.

Bioinformatic analysis of the 18 encystation-gene promoters revealed a Myb binding sequence as a signature motif for encystation-specific genes. The vast over-representation and the presence of at least

one copy of this *cis*-acting element in the 100bp upstream region of each of those genes suggests that this *cis*-acting element and the upregulated putative Myb2-like transcription factor itself are centrally involved in expression of this set of genes. Interestingly, the Myb element is absent in the genes regulated in the early (45min p.i.) response to encystation. In addition, the lack of a Myb binding element in three of the five induced genes of the UDP-GalNac synthesis pathway, shown to be upregulated later (~12 p.i.) in encystation [21], suggests difference in regulatory mechanism in genes expressed at different time points during encystation.

In addition, a comparative analysis with an analogous study performed in our lab to investigating heat stress and unfolded protein stress responses (Spycher *et al*. unpublished) revealed no significant overlap between the transcriptional response to stress and encystation. Here, we can conclude that Giardia possess a dynamic transcriptome and highly specific transcriptional regulatory mechanisms to specifically react to different environmental conditions such as fever of the host (heat), or induction of encystation.

Taken together, the data provide clear evidence that encystation is a highly specific and transcriptionally tightly controlled process and that off-target effects due to the protocol applied are considerable.

1.5 Histone acetylation and stage-specific gene expression during *G. lamblia* encystation

In the study above (1.3), we demonstrated that the transcriptional response to different stimuli is highly specific in Giardia. A dynamic transcriptome requires tight control of gene activation/silencing. The factors involved in eukaryotic transcription include the DNA templates, the RNA polymerases, general transcriptional factors, the histones and associated regulatory molecules [23]. In particular, chromatin remodeling is an important factor in regulating gene expression patterns and is defined by a combination of histone modifications also called the histone code. For example, the link between transcription activity and acetylation of N-terminal tails of the core histones is well-documented [24] and conserved from protozoa to mammals [25].

The rapid transcriptional responses to stress and differentiation stimuli is slightly at odds with the presenceof only a few transcription factors in Giardia [26]. This suggests that other regulatory elements might play dominant roles to guarantee a fast response to environmental stimuli. An *in silico* analysis performed in this study revealed a reduced, but conserved histone deacetylation machinery, with only one predicted deacetylase (HDAC). The nuclear localization of the protein further supports its identity. The presence of a single HDAC is striking in contrast with the size of the HDAC repertoire found not only in metazoans but also in unicellular protozoa, including the apicomplexan *Toxoplama gondii,* with five putative HDACs.

Global analysis of the effect on gene expression in cells treated with an HDAC inhibitor (FR235222) revealed several insights into the role of acetylation during stage differentiation in Giardia. First, we showed that histone acetylation generally decreased during stage differentiation. This is consistent with the idea that the cyst is a dormant, transcriptionally silent stage which gets reactivated only after uptake by a new host. Second, treating encysting cells with a histone deacetylase inhibitor prevented this drop in histone acetylation and consequently blocks induction of encystation. Inhibition of stage differentiation in response to HDAC inhibitors (Trichostatin A, HC-toxin) was also observed in the protozoa Entamoeba [27]. In contrast, Bougdour *et al*. [28] showed that FR235222 induces stage differentiation of the tachyzoite (rapidly replicating stage) into the bradyzoite (dormant stage) in *Toxoplasma gondii*. Although little is known about the mechanisms for expression regulation in

Apicomplexa, histone modification, particularly acetylation, is emerging as key strategy used during differentiation and stage conversion [28]. Thus, while histone hyperacetylation leads to opposite outcomes in Toxoplasma and Giardia with respect to stage differentiation, the central role for chromatin remodeling at these key junctions of the life cycles may reflect a monophyletic origin of the underlying principle. Third**,** we observed a block of CWP1 induction on the transcriptional level in encysting cells treated with FR23522. Overall, induction of nine of the identified 18 *bona fide* encystation-specific genes including all highly expressed structural proteins and Myb2 was significantly impaired in presence of FR235222. Our combined results reveal a significant overrepresentation of encystation-specific genes among those regulated by histone acetylation states. Fourth, using reporter constructs which were maintained as episomes in transgenic cells we showed that only 100 bp of flanking regions of the CWP1 genes are necessary and sufficient for conferring a FR235222-mediated inhibition of gene expression. This also works if the plasmid is integrated into a different chromosomal region between two constitutively expressed genes. On the one hand these results suggest that chromosome remodeling at the CWP loci is not the mechanism for induction of stage-specific gene expression. On the other, this fits with data suggesting that CWP genes are silenced in trophozoites through binding of a potent repressor to the short stretch upstream of encystation-specific genes [29]. Finally, we showed that FR23522 induced a specific set of genes (~2 % of the genes in Giardia) both in proliferating and encysting trophozoites. A compositional analysis of the dataset revealed a strong overrepresentation of genes coding for hypothetical and high cysteine membrane proteins (HCMP). The latter is a recently described protein family of predicted invariant surface proteins with unknown function [20].

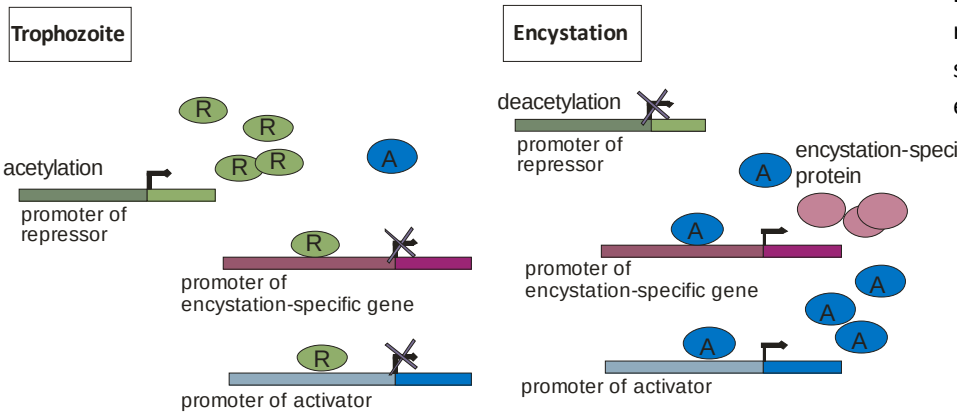
Taken together, in this first study addressing the role of histone acetylation in proliferating and encysting Giardia we find strong evidence that histone acetylation plays a key role during stage differentiation, similar to other protozoan parasites, but with clearly different mechanisms.

1.6 Model: Encystation-specific genes are induced by de-repression

As mentioned above, constitutive and regulated Giardia promoters are very short and usually contained within 100 bp or less immediately upstream of the transcriptional start codon with a small degenerate initiator sequence [30-32]. In some cases, constitutively expressed genes (e.g., tubulin) were shown to possess an A/T-rich region, with a CAAT-like element upstream of the (Inr) element required for gene expression, but normally, standard eukaryotic signals are not found in giardial promoters. Several papers presented data on regulation of CWP expression which invoked multiple *cis*-acting elements such as the Myb binding element (see Introduction 2.3). Especially, the CWP2 promoter was investigated in more detail using serial deletion of short elements (of ~10bp each) in the -10 to -64 bp upstream of the transcriptional start codon. This study gave strong evidence that a repressor might be bound to the CWP2 promoter in the trophozoite stage in the -23 to -64 region and an activator during encystation in the -10 to -23 region suggesting that CWPs are regulated via coordinated de-repression and boosting of basal transcription activity of its promoters during encystation [29].

In the course of my studies I developed solid evidence that CWP genes belong to a small set of 18 genes upregulated during encystation. We showed that all encystation-specific genes possess a common *cis*-acting element within the 100 bp upstream regions, suggesting a common regulatory mechanism. Furthermore, the global effect of a histone deacetylase inhibitor showed that hyperacetylation leads to repression of encystation and prevents induction of encystation-specific genes. The combined data suggest that decondensation through histone acetylation might be indirectly responsible for induction of encystation-specific genes.

We have constructed a model that fits all data which invokes expression of a potent repressor whose regulation is dependent on histone acetylation. Specifically, hypoacetylation of this locus in cells induced to differentiate leads to silencing of the repressor gene during encystation. We propose that the repressor competes sterically as well as quantitatively with putative activators (e.g. Myb2). Therefore, when the repressor is depleted during encystation, the activator gains access to the promoter region of encystation-specific genes and consequently leads to rapid upregulation of this gene. An activator such as Myb2 is in turn boosting its own transcription and therefore becomes quickly quantitatively dominant. In presence of a histone deacetylase inhibitor, however, hypoacetylation and consequently silencing of the repressor gene is blocked and therefore no de-repression of encystation specific genes and the activator gene can occur.



Model 2: Schematic of de-repression of encystation-specific genes during encystation

A: activator, R: repressor

2. Perspectives

In this study we gained novel insights into many aspects of the complex process of gene regulation during encystation of *G. lamblia* and obtained first evidence that histone modifications or chromatin remodeling plays a crucial role in stage conversion. To validate our model we need to characterize in detail the mechanisms that regulated activation and repression of encystation-specific genes.

1) As described above (see also PART IV manuscript 2), we observed a two wave induction of genes, an early wave at 45min p.i. and a later at 7h p.i, during early encystation. Gene encoding the known structural proteins (CWP1-3) and Myb2 are included in the second wave and all *bona fide* defined encystation-specific genes at this time point have at least one conserved Myb binding sequence in the promoter region, suggesting a coordinated regulation. Here, we formulated the hypothesis that gene expression during encystation is strictly time dependent and may be regulated by distinct factors or mechanism. To test this hypothesis, an electrophoretic mobility shift assay (EMSA) with follow up mass spectrometry analysis of the promoter regions would allow us to identify *trans*-acting factors involved in both the early and late phases of our two wave induction model during encystation.

2) To validate the hypothesis that encystation-specific genes are repressed during the trophozoite stage, the same DNA-protein interaction study of the promoter region of the *bona fide* encystation-specific genes could be used to identify a candidate repressor. If a potential repressor is once defined a localization study can be performed to confirm nuclear localization throughout trophozoit stage. Subsequently, a chromatin immunoprecipitation (ChIP) analysis of the repressor encoding region can be performed by using an antibody against acetyaletd lysine [33]. This would allow us to confirm that the genomic region of the repressor is hypoacetylated during encystation in line with the model that we

propose. Further, the by ChIP isolated DNA fragment can be sequenced to define a possible repressor binding site. Subsequently, a targeted mutagenesis analysis of the candidate repressor would reveal the amino acid residues involved in DNA binding. Overall, a detailed analysis of the candidate repressor would give great insight into the exact gene regulation mechanism during Giardia stage differentiation and would be one of the first repressor mechanism described in detail in a protozoan parasite.

3) Further, the role of histone modifications in the life cycle of Giardia could be studied in more detail by addressing questions such as:

- Are there other histone modifications than acetylation, such as methylation, important for the Giardia life cycle? A first *in silico* study revealed only few predicted histone modifying proteins in Giardia (see p. 54, manuscript 3 [33]). Here, it would be interesting to functionally analyze conserved histone modifying proteins, such as a histone methylases (HMT1, HMT2), in Giardia. First, an inhibitor study of the giardial histone methylases could shed light on the role of those enzymes in stage transition. The absence of homologous histone demethlyases in Giardia should not discourage us of testing demethylase Inhibitors too. Based on the data, expression of a dominant negative histone methylase-mutant during the life cycle of Giardia could be considered. The role of histone methylases in regulation of transcription could be system-wide investigated by microarray.

- How are the hypoacetylated genes reactivated during excystation? As a first approach, a histone acetlyase inhibitor could be used to test the hypothesis that genes must be acetylated during excystation of the cyst to form the transcriptionally more active trophozoite. Interestingly, while just one histone deacetylase (HDAC) is conserved in Giardia, five predicted histone acetyltransferases (HATs) are encoded. A localization study of tagged variants of those HATs throughout the life cycle could reveal the dynamics of their nuclear localization. Recruitment of HATs in a specific developmental stage would give us significant information on their role in stage conversion. Last but not least, if hyperacetylation occurs during excystation we expect to detect our candidate repressor upon excystation.

We have just begun to investigate the role of histone modifications/chromatin remodeling in the protozoan *G. lamblia*. It would be of interest to elucidate the extent of histone modification changes during encystation/excystation and to precisely define the role of histone modifying enzymes in the life cycle of *G. lamblia*. The stage-dependent and therefore easily inducible process offers a good platform to investigate how this modification occurs and how the cell “remembers” the exact positions of modifications while transforming to one or the other stage. Study of histone modifications and the relevant modifying enzymes in early branching eukaryotes and in simple eukaryotic cells such as *G. lamblia* will contribute in a major way to our understanding of the origins of histone codes, and how this code is implemented to help organisms adapt to various environments and progress through developmental cycles. Not in the least, research in this area also has great potential for discovery of novel therapeutic targets related to regulation of gene expression.

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**These authors contributed equally to this work, Molecular Microbiology, accepted January 2010*

Neogenesis and maturation of transient Golgi-like cisternae in a simple eukaryote
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Two distinct but interchangeable mechanisms for flipping of lipid-linked oligosaccharides
C. Alaimo, I. Catrein, **L. Morf**, C. L. Marolda, N. Callewaert, M. A. Valvano, M. F. Feldman and M. Aebi, EMBO J. 2006 Mar 8;25(5):967-76

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Glucosylceramide Synthesis is Necessary for Cell Cycle Progression, Membrane Trafficking and Stage Differentiation in *Giardia lamblia*
Saša Štefanić, Cornelia Spycher, **Laura Morf**, Gemma Fabriàs, Josefina Casas, Elisabeth Schraner, Peter Wild, Adrian B. Hehl and Sabrina Sonda

Manuscripts in preparation

Differentiation in encysting *Giardia lamblia* is determined by a small set of stage-regulated genes
L. Morf , C. Spycher, H. Rehrauer, C. Aquino Fournier, H.G. Morrison and A. B. Hehl

An unusual unfolded protein response in the absence of Ire1p in the minimized protozoan parasite *Giardia lamblia*
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Awards

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